REMARKS/ARGUMENTS

Reconsideration of this application in view of the foregoing amendments and the following remarks is respectfully requested.

With this amendment, claims 2, 3, 5, 6, 19, and 20 remain in the application. Claims 35-42 are newly submitted herein.

i. The rejection under 35 USC 102(b)/103(a) in view of McCormick

In the action, the Examiner has applied U.S. Patent No. 4,760,017 of McCormick against claims 2-6 and 19-20 as being anticipated or obvious over McCormick. The Examiner indicates that McCormick describes arabinonucleic acid and the use of a polynucleotide probe in the DNA or RNA hybridization assays. The Examiner has also noted that β-D-arabinonucleosides are used in the synthesis of the arabinonucleoside probes of McCormick. The Examiner also states that although the McCormick reference does not specifically teach that that the arabinonucleic probes can be used to induce RNaseH activity, the probes of this reference meet all of the structural limitations of Applicant's invention, particularly wherein it is drawn to an olgonucleotide consisting of β-arabinose sugars hybridizing to a single stranded RNA. Reconsideration on the following grounds by the Examiner is respectfully requested on the following grounds.

Claims 2 and 19 have been presently amended to specify that the claimed oligonucleotide compositions selectively prevent or modulate gene expression in a sequence-specific manner in a host, support for which can be found at page 17, lines 15-21 of the instant application. Claims 2 and 19 have also been presently amended to claim compositions comprising at least one of an oligonucleotide consisting of β-D-arabinose sugars hybridizing to complementary RNA to induce RNase H activity, an oligonucleotide consisting of β-D-arabinose sugars substituted at the 2' position of the sugar rings with various specified substituents and hybridizing to complementary RNA to induce RNase H activity, and an oligonucleotide consisting of β-D-arabinose sugars substituted at the 2' position of the sugar rings with halogen, alkyl, alkylhalide, alkylsulfhydryl, allyl, amino, aryl, alkoxy, or azido and hybridizing to duplex DNA/DNA or DNA/RNA to form a triple helical complex. These amendments are fully supported by the specification, for example at page 21, line 12 through to page 23, line 15, and page 22, lines 26-30 of the instant application, as well as claims 1 and 2 as originally filed in the instant application during the

international phase. Support for use of the term "complementary" when referring to RNA in the presently amended claims can be found at page 22, lines 17-2 of the instant application, and in claim 4, which has presently been cancelled.

Claim 2 has also been amended to more clearly indicate that all claimed oligonucleotide compositions are in association with an acceptable carrier.

The language of claim 3 and claim 20 has been amended to further clarify the structure of the sugar rings of the claimed oligonucleotides as being 2'-difluoro-substituted, support for which can be found at page 17, line 22 through to page 18, line 21 of the instant application.

New claims 35 through 42 have been added to further define preferred embodiments of the oligonuclotides and compositions comprising oligonucleotides of Applicant's instant invention. In particular, Applicant has added oligonucleotide claims 40-42 that correspond to composition claims 35, 38, and 3, respectively in order to fully claim Applicant's instant invention.

The dependency of claims 5 has been amended to depend from claims 2 and 3.

Claims 31-34 have been cancelled without prejudice.

The amendments to the present claims and newly submitted claims are supported by the instant application and do not present new matter.

Reconsideration by the Examiner is respectfully requested on the following grounds.

Applicant respectfully submits that there is no teaching of compositions of arabinonucleosides or derivatives thereof, including the presently claimed oligonucleotides consisting of β-D-arabinose sugars unsubstituted or substituted at 2' position of the sugar ring, for the purpose of binding RNA to elicit RNaseH activity in the reference of McCormick. Rather, McCormick teaches arabinonucleosides (ANA) can be used as probes for single-stranded DNA or RNA in the *in vitro* analysis of samples, for example, see column 3, lines 49-58. McCormick also teaches at column 3, line 59 onwards that nucleic acids to be analyzed have many sources, including clinical specimens and various microorganisms, and teaches that (our emphasis), "Extraction is one common method for collecting the nucleic acid from their source for hybridization assays. The protocol for use of the ANA probes of this invention is much like that in conventional hydridization procedures. The target DNA or RNA is first rendered single-stranded and then immobilized onto a support. The immobilized single-stranded nucleic acids are then treated with the

arabinonucleic acid probe complementary to a sequence of bases in the target." Also, as is taught in Applicant's instant application, the cellular enzyme RNaseH is known to degrade RNA of a DNA/RNA heteroduplex, and page 14 of Applicant's instant application teaches that while HIV-1 reverse transcriptase (RT)-associated RNaseH has been shown to cleave both DNA/RNA and RNA/RNA duplexes, cleavage of the latter is at least 30-fold slower and occurs only when RT is artificially arrested. Given that McCormick teaches the preparation of both single-stranded DNA and RNA, the compositions of McCormick do not necessarily teach in every instance the formation of duplexes with arabinonucleoside probes that could induce RNaseH activity, i.e., the preparation of DNA/RNA heteroduplexes.

Moreover, it is respectfully submitted that there is no explicit teaching or suggestion in McCormick that RNaseH should or would necessarily be present in the in vitro compositions containing the immobilized single-stranded nucleic acids and arabinonucleoside probes for *in vitro* analysis subsequent to the extraction and immobilization process of McCormick. Moreover, even if there were, as an artifact of the extraction and immobilization process of McCormick, a sufficient quantity of RNaseH incidentally present in a composition of McCormick containing immobilized single-stranded RNA, and that RNaseH were able to catalyze the degradation of the immobilized single-stranded RNA, it is respectfully submitted that the activation of such catalytic activity would destroy the intended functionality of McCormick's arabinonucleic acid probes. Once an arabinonucleoside probe would bind to the RNA, the resulting hybrib would be a substrate for RNaseH present in solution, resulting, in turn, in degradation of the bound RNA strand into smaller fragments. Cleavage of the bound RNA into smaller fragments would clearly destroy the intended functionality of the invention of McCormick which is the use of arabinonucleic acid probes in DNA and RNA hybridization assays for the subsequent identification of such DNA or RNA. As is described at column 3, lines 50 through to column 4, line 25 of McCormick, the successive washings to remove any unbound arabinonucleoside probe would likely likewise wash away the RNA degradation products and would not afford an immobilized RNA-ANA hybrid for subsequent labeling with an anti-arabinose antibody-label conjugate as is clearly intended.

Moreover, Applicant respectfully submits that there is nothing in McCormick that directly evidences the presence of RNaseH in the compositions of McCormick, nor are there working examples that evidence the formation of RNA-ANA duplexes eliciting RNase H activity. If a prior art reference does not expressly set forth a particular element in the claim, the reference may still anticipate if the element is inherent in the disclosure. However, to establish inherency, the extrinsic evidence must make clear that the missing descriptive matter is necessarily present in the reference, and that it would be so

recognized by persons of ordinary skill. Inherency may not be established by probabilities or possibilities, and the mere fact that a certain thing may result from a given set of circumstances is not sufficient (*Continental Can Co. v. Monsanto Co.*, 948, F.2d 1264, 1268, 20 U.S.P.Q.2d 1746, 1749 (Fed. Cir. 1991); *In re Orlrich*, 666 F.2d 578, 581, 212 U.S.P.Q. 323, 326, (C.C.P.A. 1981). Given that there is no evidence that McCormick does not teach in every instance RNA-ANA duplexes that could elicit RNase H activity as is claimed in the present amended claims, Applicant respectfully submits that the present claims are novel in view of McCormick.

Moreover, Applicant respectfully submits neither McCormick nor any other prior art has taught or suggested the use of antisense ANA's for cleaving hybridized RNA by RNase H as is claimed in the present invention. Until the instant invention, there were no known examples of uniformly *sugar-modified* oligonucleotides that elicited RNaseH activity upon association with a target RNA. Pages 9 through 20 of the instant application describes that only DNA-based oligomers (i.e., those containing 2-deoxyribose) were known in the prior art to elicit RNase H activity. This is primarily because the enzyme normally processes DNA/RNA hybrids (not RNA/RNA duplexes); and the activity/specificity of RNase H is extremely sensitive to structural changes made to the heteroduplex (e.g. modifications made to the sugar-phosphate backbone of the antisense DNA or target RNA strand). In fact, of the 60 types of modified oligomers studied during the period 1994-97 in the prior art, none apart from DNA-based oligos (PS-DNA and PS-DNA gapped oligos) were reported to activate RNase H (see review article by Sanghvi, Y. In *Comprehensive Natural Product Chemistry*; Barton, D. H. R., Nakanishi, K., Meth-Coth, O., Eds.; Elsevier Science: Oxford, UK, 1998). Thus, Applicants' discovery that a 2'-modified oligomer (such as 2'-OH or 2'F-arabinose) was capable of eliciting RNase H was totally unexpected. Therefore, the presently claimed oligonucleotides, such as oligonucleotides consisting of arabinose sugars (ANA with 2'OH groups), 2'-substituted β-D-arabinose sugars such as FANA (2'F), and 2'-difluoro-substituted sugars of the present invention represent the first examples of uniformly sugar-modified oligonucleotides that elicit RNase H activity. Applicant respectfully submits this is further evidenced by various scientific articles that evidence the surprising invention, e.g., as described in Oliver Seitz, Angew. *Chem. Int. Ed.* **1999**, 38, 23, 3466-3469; Jens Kurreck, *Eur. J. Biochem.* **270**, 1628-1644 (**2003**); and Lisbet Kvaerno and Jesper Wengel, *Chem. Commun.* **2001**, 1419-1424, copies of which are being submitted with the present response for consideration by the Examiner.

As such, Applicant respectfully submits that one of skill in the art would not have considered obvious the compositions containing the presently claimed oligonucleotides to elicit RNase H activity to selectively prevent gene expression in view of McCormick or any prior art reference made of record.

Moreover, Applicant respectfully submits that the oligonucleotides of McCormick do not fall within the Applicant's presently claimed oligonucleotides or compositions of Applicant's new claims 35-42. As such, Applicant respectfully submits that the oligonucleotides do not fall within the instant claimed invention in claims 35-42, and thus McCormick is not anticipatory with respect to these claims.

In view of the foregoing, Applicant respectfully submits that claims 2, 3, 5, 6, 19, 20, and 35-42 are directed to subject matter that is inventive and novel in view of McCormick and respectfully submit that the Applicants were the first to show the use of an oligonucleotide based on arabinose sugars for cleaving RNA via RNaseH activation. None of the prior art teaches or suggest the present invention. Withdrawal of the novelty and obviousness rejection is accordingly respectfully requested.

ii. The rejection under 35 USC 103(a) over Cheng et al. in view of Chu et al. and Meyer et al.

In the action, the Examiner has maintained the rejection in respect of claims 2-6 and 19-20 under 35 USC 103(a), and has rejected claims 31-34 under 35 USC 103(a) as being unpatentable over Cheng et al. (US Patent No. 5,646,126) in view of Chu et al. (US Patent No. 5,808,040) and Meyer et al., (US Patent no. 5,177,196) for the reasons set forth in the Official Action dated September 25, 2003.

Reconsideration of amended claims 2, 3, 5, 6, 19, and 20 and new claims 35-42 by the Examiner is respectfully requested on the following grounds.

The reference of Meyer et al. discloses novel oligonucleotide compositions formed from α-D-arabinofuranosyl nucleoside monomers. These oligonucleotides are disclosed as being useful as chemotherapeutic agents to control the expression of gene sequences or to inhibit mRNA translation. Meyer et al. does not disclose oligonucleotide compositions formed from β-arabinose units as is presently claimed, nor would it have been obvious to one having ordinary skills in the art, at the time of the invention, to arrive to the present invention from the teaching of Meyer, Jr. et al since it does not comprise any incentive to prepare oligonucleotide composition formed from β-arabinose units.

The reference of Chu et al. teaches a method for stabilizing oligonucleotides by including 2'-deoxy-2'-fluoro-arabinofuranosyl nucleosides into the oligonucleotides. Additionally, the reference of Chu et al. teaches that 2'-deoxy-2'-fluoro-β-L arabinosyluridine is a potent antiviral agent against HBV and EBV. The Examiner has indicated in the Official Action mailed June 30, 2004 that Applicant's claims do not recite that the arabinonucleoside units are 'D-nucleotide" units, as set out in Applicant's response filed April 8, 2004. Applicant has presently amended the claims to indicate that the oligonucleotides of the present invention are D-oligonucleotides comprised entirely of D-arabinonucleotide units. It is Applicant's respectful assertion that Chu et al does not disclose oligonucleotide compositions formed from β-D-arabinose units as is presently claimed, but rather consistently teaches the use of the incorporation of L-nucleosides in an oligonucleotide due to their stability against nucleases. Applicant likewise respectfully submits that it would not have been obvious to one having ordinary skills in the art at the time of the invention from the teachings of Chu to arrive to the present invention from the teaching of Chu et al., since Chu does not provide any incentive to prepare oligonucleotide composition formed from β-D-arabinose units, in place of those with L-stereochemistry, given that it would be understood by one of skill in the art that it is the unnatural L-configuration of the nucleosides of Chu confers stability to the nucleosides against nuclease degradation.

The reference of Cheng et al. describes oligonucleotides comprising 2'-deoxy, 2'-fluoro or 2'-difluoro nucleosides, wherein between 8 and 18 of said nucleosides are linked consecutively. Additionally, Cheng et al. teach that ODNs (oligonucleotides) including α and β arabinosides are included within the scope of the invention. Cheng et al. does not specifically disclose isolated oligonucleotides comprising arabinose sugars and 2'-fluoro or 2'-difluoro modified nucleosides consecutively linked in the same molecule.

The Examiner states that absent evidence to the contrary the skilled artisan would expect that if a portion of the molecules of Cheng et al. comprised a sequence that is complementary to a target RNA sequence, these molecules would interact with cellular RNA and form a complex. Applicant respectfully submits that throughout the reference of Cheng et al. is disclosed the synthesis and use of <u>duplexes</u> (not single stranded oligomers) which by virtue of their duplex structure are endowed with anticancer properties. In the Summary of the Invention at Column 5, lines 18-24, Cheng et al. states the following (our emphasis):

> *"It has been discovered in accordance of the present invention that the stable <u>duplex</u> oligonucleotides which have the structures described above in the Summary of the Invention*

and shown in FIG. 1 have significant selective cytotoxic activity against certain cancer cell lines, including some cancer cell lines which have resistance to several established anticancer agents."

Thus, Applicant respectfully submit that the mechanism of action intended by the inventors is not antisense, i.e. preventing or modulating gene expression in a sequence-specific manner as it is the case for the composition of the claimed invention. This is further described explicitly by Cheng *et al.* at column1, line 59-61 through to column 2, lines 14-19 as follows (our emphasis):

"To this date however, results with antisense oligonucleotides (ODNs) have been somewhat disappointing in terms of successful chemotherapy ... As far as the present inventors are aware, the present invention provides, for the first time, modified oligonucleotides which show anticancer activity in other than an "antisense" manner, and which show selective toxicity toward certain cancer cell lines, and to certain cancer cell lines with multiple drug resistance."

Likewise, it is the Applicant's respectful assertion that the oligomers of Cheng et al. are clearly described as and designed to be self-complementary (for example, see the ODN of Figure 5 which is described at column 7, lines 27-34 as being capable of forming substantially stable duplexes with itself and corresponding to type 1 oligomers of the invention of Cheng) in order to promote self-hybridization and duplex formation. Moreover, some of the oligomers of Cheng et al. are described as being designed to fold back upon themselves to form a hairpin structure (for example, see Figure 1, type 3). The aliphatic linkers of Cheng et al. ("hairpin turns") shown in Figure 14 are described as facilitating folding and hairpin formation. Moreover, Cheng et al. notes that when the sequence of the oligonucleotide was designed so that an imperfect duplex would form (e.g., only 8 bp of potentially 10 bp) biological activity was compromised (e.g. see column 8, lines 18-35). Column 8, lines 34-39 of Cheng et al. likewise emphasizes the requirement of the duplex structure in the ODNs of Cheng et al. and column 8, lines 36-64 of Cheng et al. provide data suggesting that the most active compounds are the most stable duplexes, whereas the inactive compounds exist as single strands. Gel electrophoresis data was used to support this conclusion (see Figure 7 of Cheng et al.).

Applicant has noted that several of the references listed on the covering page of Cheng et al. relate to "decoy" nucleic acids, which are also referred to in the art as "aptamers". Aptamers are known to those of skill in the art to generally refer to duplexes which are capable of binding proteins

and, as such, serve as "sinks" by blocking the protein from further function. For proteins involved in cellular signaling, a specific aptamer duplex interaction could ultimately modulate expression of the respective gene(s) in that pathway. On the other hand, if the aptamer duplex binds a viral protein, it might inhibit further proliferation and/or infectivity, thereby serving as a potent antiviral agent. The latter approach is the one pursued in the work by Ma et al. – which is cited under "other publications". Here RNA duplexes are designed to bind and inhibit HIV-1 viral proteins (tat). A similar approach is described by Bielinska et al., also cited under "other publications". Applicant respectfully submits that these approaches are very different from the antisense approach whereby a single stranded oligomer (not duplex) is designed to bind to mRNA (not a protein).

Applicant respectfully submits that one having ordinary skills in the art to which the invention pertains would readily understand that the oligomers of Cheng et al. are meant to be duplexes, and would not have been lead to modify the invention of Cheng et al. with the method of Chu et al., to arrive to the present invention since our oligonucleotides provide activity in an "antisense" manner, i.e. their base sequence is designed so that they (a) remain as single strands (not duplexes), and (b) are reverse complementary to the sense RNA target.

The amendments to claims as presented above are believed to overcome the Examiner's rejections under 35 U.S.C. 103(a).

No new matter is entered.

It is submitted, therefore, that the claims are in condition for allowance. Reconsideration of the Examiner's rejections is respectfully requested.

In the event that there are any questions concerning this amendment or the application in general, the Examiner is respectfully urged to telephone the undersigned so that prosecution of this application may be expedited.

Date: 11/30/04

Respectfully submitted,

David S. Resnick (Reg. No. 34,235)
NIXON PEABODY LLP
100 Summer Street
Boston, MA 02110
(617) 345-6057

Attachments: Oliver Seitz, Angew. *Chem. Int. Ed.* **1999**, 38, 23, 3466-3469;
Jens Kurreck, *Eur. J. Biochem.* **270**, 1628-1644 (**2003**);
Lisbet Kvaerno and Jesper Wengel, *Chem. Commun.* **2001**, 1419-1424,

Chemically Modified Antisense Oligonucleotides—Recent Improvements of RNA Binding and Ribonuclease H Recruitment

Oliver Seitz*

Early this year the first oligonucleotide drug, the phosphorothioate ISIS-2922 (Fomivirsen), against CMV retinitis was admitted to the market. Twenty-one years after the introduction of the antisense approach this new drug might serve as the ultimate proof of principle.[1] In the antisense concept an exogeneously added antisense oligomer selectively binds to the target mRNA and blocks the translation. A great deal of the attractiveness of this approach arises from the possibility of designing RNA binders according to the well-known Watson–Crick base-pairing rules. Thus, with such designed binders it would be feasible to virtually knock out any (pathogenic) protein at will. Phosphorothioates, the first generation of antisense oligomers, exhibit a relatively low binding affinity to the target RNA. However, the increased cellular uptake and nuclease resistance render them biological active. In addition, ribonuclease H (RNase H), an enzyme that cleaves RNA in DNA · RNA hybrids, accepts phosphorothioate · RNA hybrids and allows for the destruction of multiple copies of mRNA per antisense oligomer. Rather than providing a comprehensive overview of the progress in antisense technology, this highlight focuses on recent improvements of key elements such as RNA binding and RNase H recruitment.

Flanagan and co-workers reported on an impressive enhancement of the potency of an antisense phosphorothioate (S-AON).[2] Their 20-mer unit with only one base modification inhibited the expression of the *c-raf* gene at nanomolar concentrations. This translated into a 25 fold increase of the antisense potency relative to a previously optimized 20-mer S-AON, which is currently being tested in phase II clinical trials for the treatment of cancer. The modified heterocycle is based on a cytosine analogue synthesized by Lin and Matteucci.[3] A tricyclic phenoxazine **2** serves as a rigid scaffold for the attachment of groups suitable for further interactions with nucleobases (Figure 1). By appending an aminoethyloxy tether, the phenoxazine was armed with a strong hydrogen bond donor that would recognize both the Watson–Crick and the Hoogsteen sites of guanine, and was thus termed a G-clamp (**3**; Figure 2). The cytosine analogues

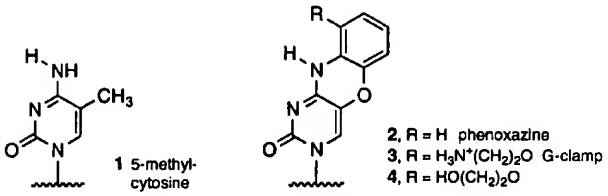

Figure 1. Cytosine analogues used in hybridization experiments (Table 1) by Lin and Matteucci.[3]

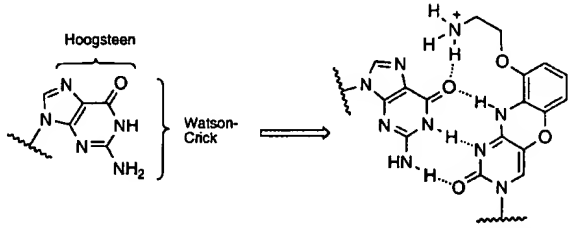

Figure 2. Proposed interaction of the G-clamp with the Watson–Crick and the Hoogsteen sites of guanosine.

shown in Figure 1 were incorporated into antisense oligonucleotides (AON) and hybridized to the complementary oligodeoxynucleotide (ODN). The melting temperature T_M, a measure of the thermodynamic stability of double stranded ODNs, were determined by analysis of the temperature-dependent UV absorbance. The G-clamp-containing AON **3** displayed a dramatically enhanced stability (Table 1), while

Table 1. Analysis of the melting temperatures of different single phenoxazine-modified AONs hybridized to matched and single-mismatched ODNs.[a]

	T_M [°C] (ΔT_M [°C])			
X	**Y** = G[b]	**Y** = A[c]	**Y** = T[c]	**Y** = C[c]
1 (5-Me-C)	50.5	32.0 (−18.5)	30.0 (−20.5)	29.0 (−21.5)
2 (phenoxazine)	57.0 (+6.5)	44.5 (−12.5)	42.0 (−15.0)	33.0 (−24.0)
3 (G-clamp)	68.5 (+18.0)	45.5 (−23.0)	41.0 (−27.5)	40.0 (−28.5)
4	51.5 (+1.0)	–	–	–

[a] Buffer: 0.14 M KCl, 5 mM Na_2HPO_4, 1 mM $MgCl_2$, pH 7.2. Test AON: 5′-TCTC**X**CTCTC-3′. Target ODN: 3′-AGAG**Y**GAGAGA$_5$-5′. [b] Matched ODNs: ΔT_M relative to **X** = **1**. [c] Mismatched ODNs: ΔT_M relative to **Y** = G.

the AON with the weakly hydrogen bonding hydroxyl group (phenoxazine **4**) replacing the amino group in **3** showed a very similar T_M to the parent 5-methylcytosine **1**. In order to rule out any nonspecific effects that might be caused by ionic interactions, the authors studied the dependence of the T_M on the salt concentration. Attractive Coulomb interactions would normally be weakened at high ionic strength. However, the increased affinity of the G-clamp-modified AONs relative to the 5-methylcytosine was independent of the salt concentration (ΔT_M = 16–18 °C in the range of 0.014–1.4 M KCl), which provided further support for the proposed specific hydrogen binding. Most importantly, the G-clamp AON conferred an enhanced mismatch specificity relative to

[*] Dr. Oliver Seitz
Institut für Organische Chemie der Universität
Richard-Willstätter-Allee 2, D-76128 Karlsruhe (Germany)
Fax: (+49) 721-608-4825
E-mail: seitz@ochhades.chemie.uni-karlsruhe.de

  1433-7851/99/3823-3466 $ 17.50+.50/0

5-methylcytosine as determined by the comparison of the T_M values of matched versus mismatched hybridization (Table 1).

The greatly increased affinity and specificity of the base-modified G-clamp was confirmed in recent in vivo studies.[2] African green monkey kidney cells (CV-1 cells) were transfected with a plasmid that contained the human cyclin-dependent kinase inhibitor $p27^{kip1}$ and treated with a cytofectin and the antisense phosphorothioate (S-AON). The G-clamp (S-AON) **9** (Table 2) inhibited $p27^{kip1}$ expression by

Table 2. RNase H activity of the posphorothioates **7**, **8**, and **9**.

Compound	Sequence	Base modifications	RNase H cleavage [%][a]
7	5'-TGGCTCTCCTGCGCC-3'	none	21.7
8	5'-5GG656566 5G6G66-3'	5-propinyluracil/cytosine	4.9
9	5'-TGGCTCTC3TGCGCC-3'	G-clamp	17.4

[a] The % cleavage of complementary radioactively labeled RNA 5 min after treatment with nuclear extracts of HeLa cells (source of human RNase H).

96% at 30 nM, as measured by Western Blotting of cellular extracts. At this concentration the previously most potent S-AON **8**, which contains 11 C5-propinyl-modified bases (**5** and **6** in Figure 3) showed 67% inhibition of $p27^{kip1}$ levels. A comparison of the IC_{50} values revealed that the mono substituted strand **9** was three times more potent than the

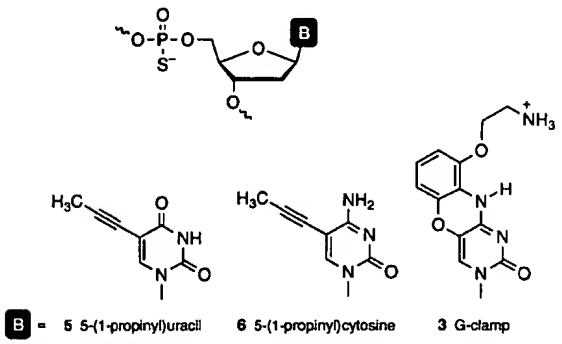

Figure 3. Nucleobase analogues incorporated in antisense agents (Table 2) by Flanagan and co-workers.[2]

multiply substituted strand **8**. The control S-AON **7** failed to demonstrate any inhibitory activity under these conditions. The sequence specificity of the G-clamp S-AON **9** was tested by transfecting the CV-1 cells with the wild-type p27 plasmid or a single base pair mutant. This mutation changed the complementary guanine in the transcribed mRNA to a uracil group. The G-clamp S-AON **9** inhibited the expression of the single base pair mutant $p27^{kip1}$ with a fivefold decrease in the IC_{50} value relative to the wild-type $p27^{kip1}$. In contrast, the inhibition of wild-type and mutant $p27^{kip1}$ was almost identical when the C5-propinyl-substituted S-AON **8** was used. The increased antisense activity of the G-clamp S-AON **9** was even more obvious when gel-shift assays were performed. The less potent C5-propinyl-substituted S-AON **8** had an affinity for the RNA target that was almost twice as high as **9**. Thus, differential induction of RNase H was expected to play an important role. The RNase H catalyzed cleavage assay demonstrated that the G-clamp S-AON · RNA heteroduplex was degraded 3.5 times more rapidly than the C5-propinyl substituted S-AON · RNA heteroduplex, and almost as rapidly as the unmodified duplex with **7** (Table 2). These results indicate that RNase H activation appears to be of major importance for antisense activity.

Nearly all nuclease-resistant antisense oligonucleotides currently in clinical trials contain modified phosphodiester backbones. However, the alteration of the natural D-2-deoxyribose itself can also provide AONs with increased stability against nuclease degradation. Unfortunately, none of the uniformly sugar-modified AONs were able to induce mRNA cleavage by RNase H catalysis. Damha and co-workers reported on a class of RNase H competent AONs based on arabinose, the 2-epimer of ribose.[4] It was demonstrated that substituting the 2'-OH group of the arabinonucleic acids (ANA) with fluorine yielded 2'-deoxy-2'-fluoro-β-D-arabinonucleic acids (2'F-ANA) with a markedly enhanced affinity towards complementary RNA (Figure 4). For example, the melting temperatures of the RNA heteroduplexes with 2'F-ANA **10** and **14** were significantly increased relative

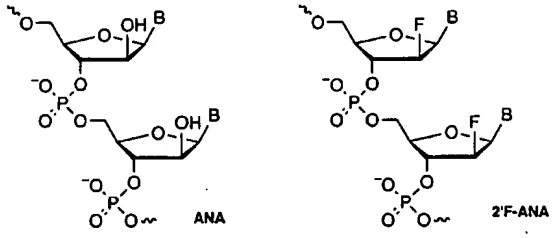

Figure 4. Chemical structure of arabino nucleic acids (ANA) and 2'-deoxy-2'-fluoroarabinonucleic acids (2'F-ANA). (B = nucleobase).

to the hybrids formed with the ANAs **11** and **15** or with the DNA strands **12** and **16** (Table 3). The higher T_M value of the 2'F-ANA · RNA heteroduplexes with **10** and **14** relative to the corresponding hybrids with the phosphorothioates (S-AON) **13** and **17** is also worth mentioning. In addition, the circular dichroism (CD) spectra of the ANA · RNA and 2'F-ANA · RNA duplexes closely resembled that of the parent DNA · RNA duplex, which suggests the common existence of an A-type helix. The specific recognition of the A-like helix of

Table 3. Analysis of the melting temperatures of the arabinonucleic acid duplexes with RNA and control DNA · RNA and S-ODN · RNA duplexes.[a]

AON sequence	Backbone modification	T_M [°C]
5'-TTTTTTTTTTTTTTTTTTTT-3'	2'F-ANA, **10**	44
	ANA, **11**	[b]
	DNA, **12**	39
	S-DNA, **13**	21
5'-TTATATTTTTCTTTCCC-3'	2'F-ANA, **14**	65
	ANA,[c] **15**	32
	DNA, **16**	51
	S-DNA, **17**	38

[a] Buffer: 0.14 M KCl, 5 mM Na_2HPO_4, 1 M $MgCl_2$, pH 7.2. [b] Not detectable. [c] Contained uracil instead of thymine.

DNA · RNA duplexes by RNase H is assumed to induce the selective hydrolysis of DNA · RNA duplexes even in the presence of RNA · RNA or DNA · DNA duplexes. In fact, similarly to the AON **12** and the S-AON **13**, the 2′F-ANA **10** was able to induce a RNase H catalyzed cleavage of the radioactively labeled RNA target. Neither the duplexes of the epimeric 2′-deoxy-2′-fluororibonucleic acids (2′F-RNA) nor the corresponding duplexes with RNA or RNA alone were attacked. Interestingly, ANA · RNA duplexes were poor substrates for RNase H, a result that was attributed to the low stability of the duplex. The 2′F-ANAs are the first class of uniformly sugar-modified antisense oligonucleotides that display an enhanced affinity for their RNA target and fully maintain the ability to induce RNase H. However, it has to be mentioned that this criterion is also met by antisense oligomers consisting of a non-uniform backbone. For example, chimera of DNA and peptide nucleic acids (PNA) are also substrates for RNase H.[5]

Although the importance of RNase H induction had been emphasized, it is still a matter of debate as to whether high affinity binding to the mRNA alone might be sufficient enough to inhibit translation. A marked increase of the RNA binding affinity and the nuclease resistance would be of pivotal significance. Remarkably, stable pairing with RNA was reported for modifications with altered internucleotide linkages such as 3′-thioformacetal,[6] methylene(methylimino) (MMI),[7] and methylene amide spacers.[8] Similiarly, sugar-modified oligomers such as 2′-O-alkylribonucleosides,[9] hexitolnucleosides,[10] or α-nucleosides[11] confer high-affinity RNA binding. Successful examples in which the entire ribose phosphate backbone has been replaced by an artificial backbone include morpholinophosphorodiamidates[12] and peptide nucleic acids.[13] An alternative approach, introduced by Leumann et al., makes use of rigidified ODN analogues.[14] The restricted conformational flexibility eventually reduces the pairing entropy ΔS and this translates into a more favorable standard enthalpy ΔG of duplex formation. In this regard, several base-pairing systems were reported.[15] The most recent example of a conformationally restricted Watson–Crick base-pairing system was published by Wengel and co-workers, who introduced the locked nucleic acids (LNA; Figure 5).[16] The methylene bridge that connects the 2′-oxygen with the 4′-carbon atom locks the sugar in the C-3′-endo conformation typical of the A-type RNA · RNA duplex,

Figure 5. Chemical structure of locked nucleic acids (LNA) and proposed locked sugar moiety in the puckered C-3′-*endo* conformation.

as demonstrated by the CD spectrum of a LNA · RNA duplex.[17] The hybridization of the mixed-sequence all-LNA nonamer **19** with the complementary DNA nonamer yielded a duplex with an unprecedented thermodynamic stability. The T_M value was increased by 36 °C relative to the corresponding DNA · DNA duplex with **18** (Table 4).[18] The **19** · RNA hybrid displayed an even higher increase of the thermal stability (ΔT_M of 46 °C) relative to the DNA · RNA duplex. The introduction of a single base pair mismatch resulted in a decrease of the T_M value by 12–14 °C, a satisfactory selectivity that accounts for the very high binding affinity of LNA for the DNA or RNA targets. To date, LNA · LNA hybridization constitutes the most thermally stable nucleic acid type duplex system as demonstrated by the T_M of 93 °C for the duplex of **19** with its complementary LNA strand.[17]

Table 4. Analysis of the melting temperatures of LNA duplexes with DNA, RNA, and LNA as well as control DNA duplexes with DNA and RNA.[a]

Compound	AON sequence	AON type	Complementary ODN	T_M [°C] (ΔT_M [°C])
18	5′-d(GTG ATATGC)-3′	DNA	DNA	28 (–)
			RNA	28 (–)
19	5′-GTG ATATG^{Me}C-3′	LNA	DNA	64 (36)
			RNA	74 (46)
			LNA	93[b] (> 65)

[a] Buffer: 0.1 M NaCl, 10 mM Na_2HPO_4, pH 7.0. [b] A low salt buffer (1 mM Na_2HPO_4, pH 7.0) had to be used since duplex dissociation was not detectable in the standard buffer.

The examples presented above indicate that key elements of antisense technology such as RNA binding affinity and selectivity, RNase H induction as well as nuclease resistance can be improved. The employment of the various strategies in concert certainly holds much promise for the development of a new generation of antisense agents, demonstrated best by the successful combination of the phosphorothioate methodology with the modified G-clamp base.

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Keywords: antisense agents · nucleic acids · nucleobases · ribonucleases · translation

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REVIEW ARTICLE

Antisense technologies

Improvement through novel chemical modifications

Jens Kurreck

Institut für Chemie-Biochemie, Freie Universität Berlin, Germany

Antisense agents are valuable tools to inhibit the expression of a target gene in a sequence-specific manner, and may be used for functional genomics, target validation and therapeutic purposes. Three types of anti-mRNA strategies can be distinguished. Firstly, the use of single stranded antisense-oligonucleotides; secondly, the triggering of RNA cleavage through catalytically active oligonucleotides referred to as ribozymes; and thirdly, RNA interference induced by small interfering RNA molecules. Despite the seemingly simple idea to reduce translation by oligonucleotides complementary to an mRNA, several problems have to be overcome for successful application. Accessible sites of the target RNA for oligonucleotide binding have to be identified, antisense agents have to be protected against nucleolytic attack, and their cellular uptake and correct intracellular localization have to be achieved. Major disadvantages of commonly used phosphorothioate DNA oligonucleotides are their low affinity towards target RNA molecules and their toxic side-effects. Some of these problems have been solved in 'second generation' nucleotides with alkyl modifications at the 2′ position of the ribose. In recent years valuable progress has been achieved through the development of novel chemically modified nucleotides with improved properties such as enhanced serum stability, higher target affinity and low toxicity. In addition, RNA-cleaving ribozymes and deoxyribozymes, and the use of 21-mer double-stranded RNA molecules for RNA interference applications in mammalian cells offer highly efficient strategies to suppress the expression of a specific gene.

Keywords: antisense-oligonucleotides; deoxyribozymes; DNA enzymes; locked nucleic acids; peptide nucleic acids; phosphorothioates; ribozymes; RNA interference; small interfering RNA.

Introduction

The potential of oligodeoxynucleotides to act as antisense agents that inhibit viral replication in cell culture was discovered by Zamecnik and Stephenson in 1978 [1]. Since then antisense technology has been developed as a powerful tool for target validation and therapeutic purposes. Theoretically, antisense molecules could be used to cure any disease that is caused by the expression of a deleterious gene, e.g. viral infections, cancer growth and inflammatory diseases. Though rather elegant in theory, antisense approaches have proven to be challenging in practical applications.

Correspondence to J. Kurreck, Institut für Chemie-Biochemie, Freie Universität Berlin, Thielallee 63, 14195 Berlin, Germany. Fax: + 49 30 83 85 64 13, Tel.: + 49 30 83 85 69 69, E-mail: jkurreck@chemie.fu-berlin.de

Abbreviations: AS, antisense; CeNA, cyclohexene nucleic acid; CMV, cytomegalovirus; FANA, 2′-deoxy-2′-fluoro-β-D-arabino nucleic acid; GFP, green fluorescence protein; HER, human epidermal growth factor; ICAM, intercellular adhesion molecule; LNA, locked nucleic acid; MF, morpholino; NP, N3′-P5′ phosphoroamidates; ON, oligonucleotide; PNA, peptide nucleic acid; PS, phosphorothioate; RISC, RNA-induced silencing complex; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; tc, tricyclo; TNF, tumor necrosis factor.

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In the present review, three types of anti-mRNA strategies will be discussed, which are summarized in Fig. 1. This scheme also demonstrates the difference between antisense approaches and conventional drugs, most of which bind to proteins and thereby modulate their function. In contrast, antisense agents act at the mRNA level, preventing its translation into protein. Antisense-oligonucleotides (AS-ONs) pair with their complementary mRNA, whereas ribozymes and DNA enzymes are catalytically active ONs that not only bind, but can also cleave, their target RNA. In recent years, considerable progress has been made through the development of novel chemical modifications to stabilize ONs against nucleolytic degradation and enhance their target affinity. In addition, RNA interference has been established as a third, highly efficient method of suppressing gene expression in mammalian cells by the use of 21–23-mer small interfering RNA (siRNA) molecules [2].

Efficient methods for gene silencing have been receiving increased attention in the era of functional genomics, since sequence analysis of the human genome and the genomes of several model organisms revealed numerous genes, whose function is not yet known. As Bennett and Cowsert pointed out in their review article [3] AS-ONs combine many desired properties such as broad applicability, direct utilization of sequence information, rapid development at low costs, high probability of success and high specificity compared to alternative technologies for gene functionalization and target validation. For example, the widely used approach to generate knock-out animals to gain information about

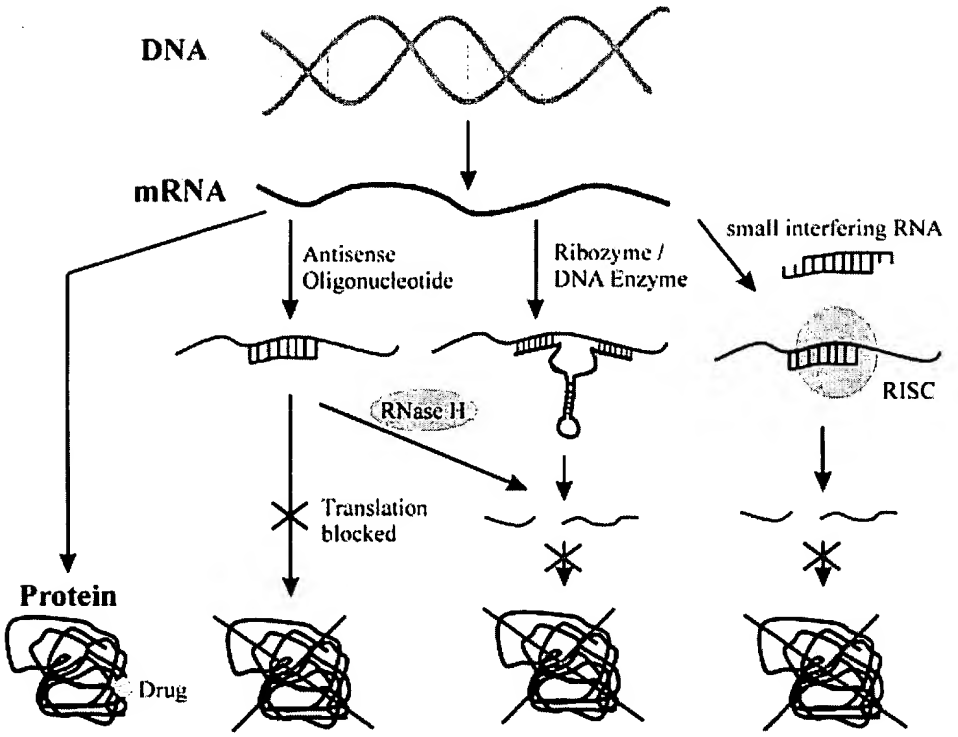

Fig. 1. Comparison of different antisense strategies. While most of the conventional drugs bind to proteins, antisense molecules pair with their complementary target RNA. Antisense-oligonucleotides block translation of the mRNA or induce its degradation by RNase H, while ribozymes and DNA enzymes possess catalytic activity and cleave their target RNA. RNA interference approaches are performed with siRNA molecules that are bound by the RISC and induce degradation of the target mRNA.

the function of genes *in vivo* is time-consuming, expensive, labor intensive and, in many cases, noninformative due to lethality during embryogenesis. In these cases, antisense technologies offer an attractive alternative to specifically knock down the expression of a target gene. Mouse E-cadherin (–/–) embryos, for example, fail to form the blastocoele, resulting in lethality in an early stage of embryogenesis, but AS-ONs, when administered in a later stage of development, were successfully employed to investigate a secondary role of E-cadherin [4]. Another advantage of the development of AS-ONs is the opportunity to use molecules for therapeutic purposes, which have been proven to be successful in animal models.

It should, however, be mentioned that it was questioned whether antisense strategies kept the promises made more than 20 years ago [5]. As will be described in detail below, problems such as the stability of ONs *in vivo*, efficient cellular uptake and toxicity hampered the use of AS agents in many cases and need to be solved for their successful application. In addition, nonantisense effects of ONs have led to misinterpretations of data obtained from AS experiments. Therefore, appropriate controls to prove that any observed effect is due to a specific antisense inhibition of gene expression are another prerequisite for the proper use of AS molecules.

Antisense-oligonucleotides

AS-ONs usually consist of 15–20 nucleotides, which are complementary to their target mRNA. As illustrated in Fig. 2, two major mechanisms contribute to their antisense activity. The first is that most AS-ONs are designed to activate RNase H, which cleaves the RNA moiety of a DNA·RNA heteroduplex and therefore leads to degradation of the target mRNA. In addition, AS-ONs that do not

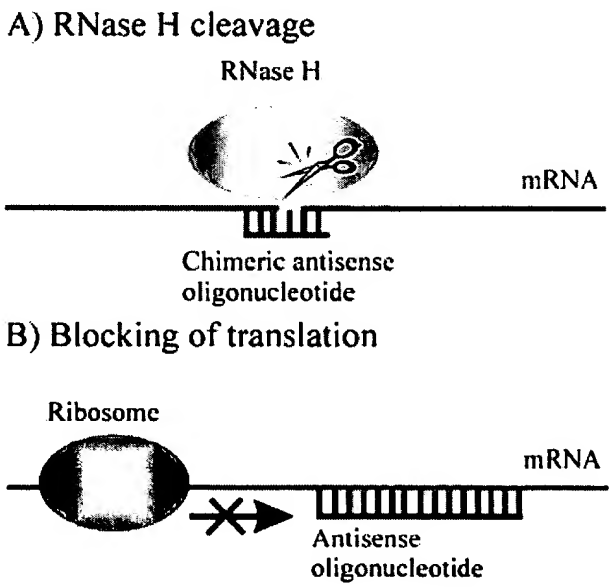

Fig. 2. Mechanisms of antisense activity. (A) RNase H cleavage induced by (chimeric) antisense-oligonucleotides. (B) Translational arrest by blocking the ribosome. See the text for details.

induce RNase H cleavage can be used to inhibit translation by steric blockade of the ribosome. When the AS-ONs are targeted to the 5′-terminus, binding and assembly of the translation machinery can be prevented. Furthermore, AS-ONs can be used to correct aberrant splicing (see below).

Long RNA molecules form complex secondary and tertiary structures and therefore the first task for a successful antisense approach is to identify accessible target sites of the mRNA. On average, only one in eight AS-ONs is thought to bind effectively and specifically to a certain target mRNA [6], but the percentage of active AS-ONs is known to vary from one target to the next. It is therefore possible to simply test a number of ONs for their antisense efficiency, but more sophisticated approaches are known for a systematic optimization of the antisense effect.

Computer-based structure models of long RNA molecules are unlikely to represent the RNA structure inside a living cell, and to date are only of limited use for the design of efficient AS-ONs. Therefore, a variety of strategies have been developed for this purpose (reviewed in [7]). The use of random or semirandom ON libraries and RNase H, followed by primer extension, has been shown to reveal a comprehensive picture of the accessible sites [8,9]. A nonrandom variation of this strategy was developed in which target-specific AS-ONs were generated by digestion of the template DNA [10]. A rather simple and straightforward method providing comparable information about the structure of the target RNA is to screen a large number of specific ONs against the transcript in the presence of RNase H and to evaluate the extent of cleavage induced by individual ONs [11]. The most sophisticated approach reported so far is to design a DNA array to map an RNA for hybridization sites of ONs [12]. Because mRNA structures in biological systems are likely to differ from the structure of *in vitro* transcribed RNA molecules, and because RNA-binding proteins shield certain target sites inside cells, screening of ON efficiency in cell extracts [13] or in cell culture might be advantageous (e.g. [14,15]).

When designing ONs for antisense experiments, several pitfalls should be avoided [6]. AS-ONs containing four contiguous guanosine residues should not be employed, as they might form G-quartets via Hoogsteen base-pair formation that can decrease the available ON concentration and might result in undesired side-effects. Modified guanosines (for example 7-deazaguanosine, which cannot form Hoogsteen base pairs) may be used to overcome this problem.

ONs containing CpG motifs should be excluded for *in vivo* experiments, because this motif is known to stimulate immune responses in mammalian systems. The CG dinucleotide is more frequently found in viral and bacterial DNA than in the human genome, suggesting that it is a marker for the immune system to signify infection. Coley Pharmaceuticals even makes use of CG-containing ONs as immune stimulants for treating cancer, asthma and infectious diseases in clinical trials [16].

Another important step for the development of an antisense molecule is to perform a database search for each ON sequence to avoid significant homology with other mRNAs. Furthermore, control experiments should be carried out with great care in order to prove that any observed effect is due to a specific antisense knockdown of the target mRNA. A number of types of control ONs have been used for antisense experiments: random ONs, scrambled ONs with the same nucleotide composition as the AS-ON in random order, sense ONs, ONs with the inverted sequence or mismatch ONs, which differ from the AS-ON in a few positions only.

In the following sections, properties of modified AS-ONs and recent advances obtained with novel DNA and RNA analogs will be discussed in more detail. Subsequently, strategies to mediate efficient cellular uptake of oligonucleotides and results of clinical trials will be described.

Antisense-oligonucleotide modifications

One of the major challenges for antisense approaches is the stabilization of ONs, as unmodified oligodeoxynucleotides are rapidly degraded in biological fluids by nucleases. A vast number of chemically modified nucleotides have been used in antisense experiments. In general, three types of modifications of ribonucleotides can be distinguished (Fig. 3): analogs with unnatural bases, modified sugars (especially at the 2′ position of the ribose) or altered phosphate backbones.

A variety of heterocyclic modifications have been described, which can be introduced into AS-ONs to strengthen base-pairing and thus stabilize the duplex between AS-ONs and their target mRNAs. A comprehensive review dealing with base-modified ONs was published previously by Herdewijn [17]. Because only a relatively small number of these ONs have been investigated *in vivo*, little is known about their potential as antisense molecules and their possible toxic side-effects. Therefore, the present review will focus on ONs with modified sugar moieties and phosphate backbones.

'First generation' antisense-oligonucleotides

Phosphorothioate (PS) oligodeoxynucleotides are the major representatives of first generation DNA analogs that are the best known and most widely used AS-ONs to date (reviewed in [18]). In this class of ONs, one of the nonbridging oxygen atoms in the phophodiester bond is replaced by sulfur (Fig. 4). PS DNA ONs were first synthesized in the 1960s by Eckstein and colleagues [19] and were first used as AS-ONs for the inhibition of HIV

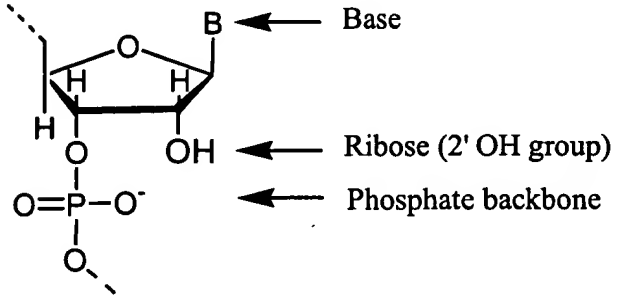

Fig. 3. Sites for chemical modifications of ribonucleotides. B denotes one of the bases adenine, guanine, cytosine or thymine.

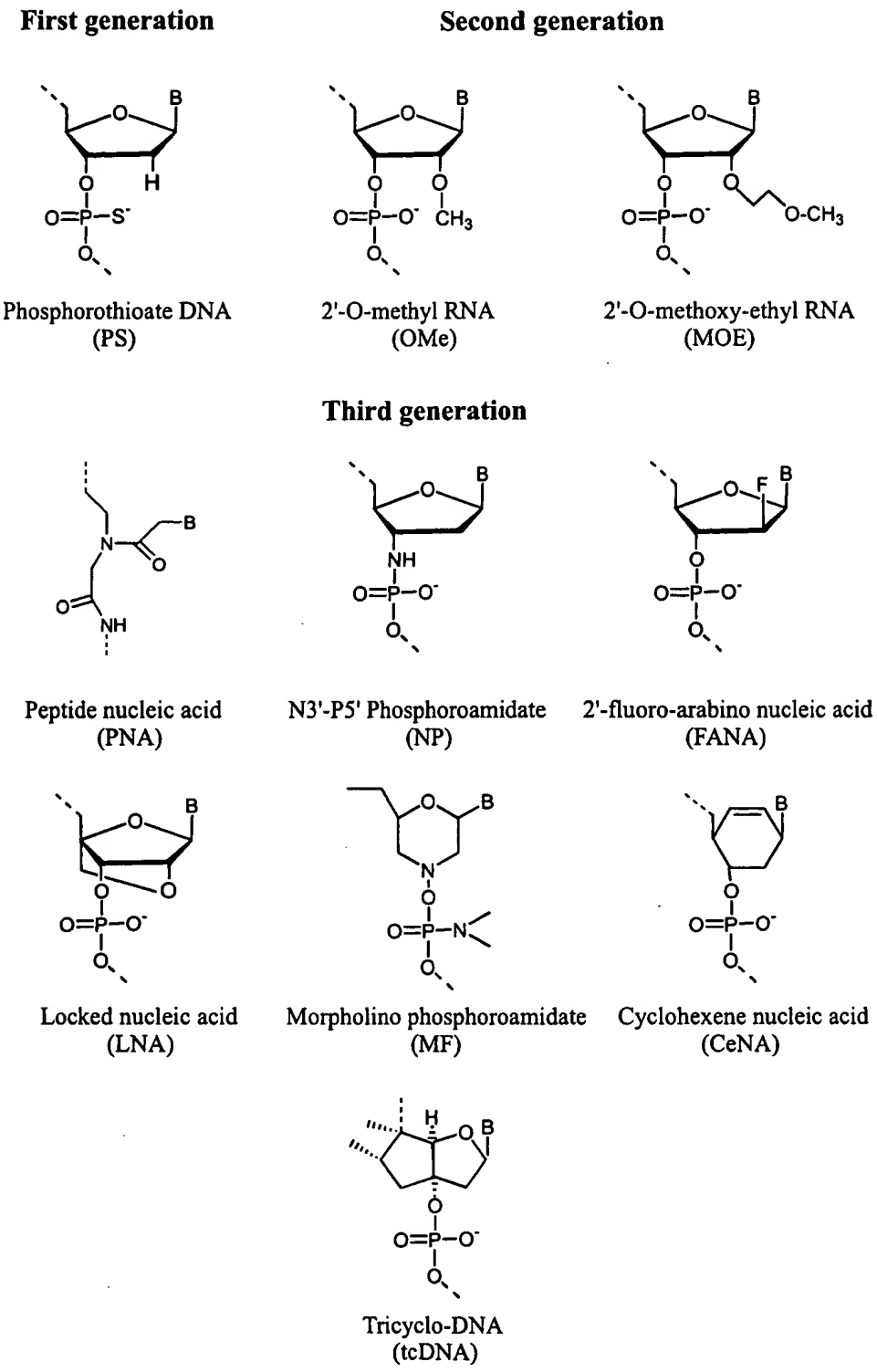

Fig. 4. Nucleic acid analogs discussed in this review. B denotes one of the bases adenine, guanine, cytosine or thymine.

replication by Matsukura and coworkers [20]. As described below, these ONs combine several desired properties for antisense experiments, but they also possess undesirable features.

The introduction of phosphorothioate linkages into ONs was primarily intended to enhance their nuclease resistance. PS DNAs have a half-life in human serum of approximately 9–10 h compared to ≈1 h for unmodified oligodeoxynucleotides [21–23]. In addition to nuclease resistance, PS DNAs form regular Watson–Crick base pairs, activate RNase H, carry negative charges for cell delivery and display attractive pharmacokinetic properties [24].

The major disadvantage of PS oligodeoxynucleotides is their binding to certain proteins, particularly those that interact with polyanions such as heparin-binding proteins (e.g. [25–27]). The reason for this nonspecific interaction is not yet fully understood, but it may cause cellular toxicity [reviewed in 28]. After PS DNA treatment of primates, serious acute toxicity was observed as a result of a transient activation of the complement cascade that has in some cases led to cardiovascular collapse and death. In addition, the clotting cascade was altered after the administration of PS DNA ONs. The lower doses of PS oligodeoxynucleotide used for clinical trials in humans, however, were generally well tolerated, as will be discussed below. Furthermore, the seemingly negative property of PS DNA ONs to interact with certain proteins proved to be advantageous for the pharmacokinetic profile. Their binding to plasma proteins protects them from filtration and is responsible for an increased serum half-life [28].

Another shortcoming of PS DNAs is their slightly reduced affinity towards complementary RNA molecules in comparison to their corresponding phosphodiester oligodeoxynucleotide. The melting temperature of a heteroduplex is decreased by approximately 0.5 °C per nucleotide. This weakness is, in part, compensated by an enhanced specificity of hybridization found for PS ONs compared to unmodified DNA ONs [24].

'Second generation' antisense-oligonucleotides

The problems associated with phosphorothioate oligodeoxynucleotides are to some degree solved in second generation ONs containing nucleotides with alkyl modifications at the 2′ position of the ribose. 2′-*O*-methyl and 2′-*O*-methoxy-ethyl RNA (Fig. 4) are the most important members of this class. AS-ONs made of these building blocks are less toxic than phosphorothioate DNAs and have a slightly enhanced affinity towards their complementary RNAs [23,29].

These desirable properties are, however, counterbalanced by the fact that 2′-*O*-alkyl RNA cannot induce RNase H cleavage of the target RNA. Mechanistic studies of the RNase H reaction revealed that the correct width of the minor groove of the AS-ON·RNA duplex (closer to A-type rather than B-type), flexibility of the AS-ON and availability of the 2′-OH group of the RNA are required for efficient RNase H cleavage [30].

Because 2′-*O*-alkyl RNA ONs do not recruit RNase H, their antisense effect can only be due to a steric block of translation (see above). The effectiveness of this mechanism was first shown in 1997, when the expression of the intercellular adhesion molecule 1 (ICAM-1) could be inhibited efficiently with an RNase H-independent 2′-*O*-methoxy-ethyl-modified AS-ON that was targeted against the 5′-cap region [31]. This effect was probably due to selective interference with the formation of the 80S translation initiation complex.

Another approach, for which the ON must avoid activation of RNase H, is an alteration of splicing. In contrast to the typical role for AS-ONs, in which they are supposed to suppress protein expression, blocking of a splice site with an AS-ON can increase the expression of an alternatively spliced protein variant. This technique is being developed to treat the genetic blood disorder β-thalassemia. In one form of this disease, a mutation in intron 2 of the β-globin gene causes aberrant splicing of the pre-mRNA and, as a consequence, β-globin deficiency. A phosphorothioate 2′-*O*-methyl oligoribonucleotide that does not induce RNase H cleavage was targeted to the aberrant splice site and restored correct splicing, generating correct β-globin mRNA and protein in mammalian cells [32].

For most antisense approaches, however, target RNA cleavage by RNase H is desired in order to increase antisense potency. Therefore, 'gapmer technology' has been developed. Gapmers consist of a central stretch of DNA or phosphorothioate DNA monomers and modified nucleotides such as 2′-*O*-methyl RNA at each end (indicated by red and yellow regions of the ON in Fig. 2B). The end blocks prevent nucleolytic degradation of the AS-ON and the contiguous stretch of at least four or five deoxy residues between flanking 2′-*O*-methyl nucleotides was reported to be sufficient for activation of *Escherichia coli* and human RNase H, respectively [29,33,34].

The use of gapmers has also been suggested as a solution for another problem associated with AS-ONs, the so-called 'irrelevant cleavage' [5]. The specificity of an AS-ON is reduced by the fact that it nests a number of shorter sequences. A 15-mer, for example, can be viewed as eight overlapping 8-mers, which are sufficient to activate RNase H. Each of these 8-mers will occur several times in the genome and might bind to nontargeted mRNAs and induce their cleavage by RNase H. This theoretical calculation became relevant for a 20-mer phosphorothioate oligodeoxyribonucleotide targeting the 3′-untranslated region of PKC-α. Unexpectedly, PKC-ζ was codown-regulated by the ON, probably due to irrelevant cleavage caused by a contiguous 11-base match between the ON and the PKC-ζ mRNA. Gapmers with a central core of six to eight oligodeoxynucleotides and nucleotides unable to recruit RNase H at both ends can be employed to eliminate irrelevant cleavage, as they will only induce RNase H cleavage of one target sequence.

'Third generation' antisense-oligonucleotides

In recent years a variety of modified nucleotides have been developed (Fig. 4) to improve properties such as target affinity, nuclease resistance and pharmacokinetics. The concept of conformational restriction has been used widely to enhance binding affinity and biostability. In analogy to the previous terms 'first generation' for phosphorothioate DNA and 'second generation' for 2′-*O*-alkyl-RNA, these novel nucleotides will subsequently be subsumed under the term 'third generation' antisense agents. DNA and RNA analogs with modified phosphate linkages or riboses as well as nucleotides with a completely different chemical moiety substituting the furanose ring have been developed, as will be described below. Due to the limited space, only a few promising examples of the vast body of novel modified nucleotides with improved properties can be discussed here, although further modifications may prove to have a great potential as antisense molecules.

Peptide nucleic acids (PNAs). Peptide nucleic acids (PNAs) belong to the first and most intensively studied DNA analogs besides phosphorothioate DNA and 2′-*O*-alkyl RNA [reviewed in 35–37]. In PNAs the deoxyribose phosphate backbone is replaced by polyamide linkages. PNA was first introduced by Nielsen and coworkers in 1991 [38] and can now be obtained commercially, e.g. from Applied Biosystems (Foster City, CA, USA). PNAs have favorable hybridization properties and high biological stability, but do not elicit target RNA cleavage by RNase H. Additionally, as they are electrostatically neutral molecules, solubility and cellular uptake are serious problems that have to be overcome for the usage of PNAs as antisense agents to become practical. Improved intracellular delivery could be obtained by coupling PNAs to negatively charged oligomers, lipids or certain peptides that are efficiently internalized by cells [summarized in 35,37].

In one of the latest and most convincing *in vivo* studies, PNAs (as well as several other modified ONs) were used to correct aberrant splicing in a transgenic mouse model [39]. The ONs were directed against a mutated intron of the human β-globin gene that interrupted the gene encoding enhanced green fluorescent protein (GFP). Only in the presence of systemically delivered AS-ONs was the functional GFP expressed. Interestingly, PNAs linked to four lysines at the C-terminus were the most effective of the AS-ONs investigated, whereas a 2′-*O*-methoxy-ethyl ON had a slightly lower activity in all tissues except the small intestine. Morpholino (MF) ONs were significantly less effective while PNA with only one lysine was completely inactive, indicating that the four-lysine tail is essential for antisense activity of PNAs *in vivo*.

According to the *in vivo* studies performed to date, PNAs seem to be nontoxic, as they are uncharged molecules with low affinity for proteins that normally bind nucleic acids. The greatest potential of PNAs, however, might not be their use as antisense agents but their application to modulate gene expression by strand invasion of chromosomal duplex DNA [37].

N3′-P5′ phosphoroamidates (NPs). N3′-P5′ phosphoroamidates (NPs) are another example of a modified phosphate backbone, in which the 3′-hydroxyl group of the 2′-deoxyribose ring is replaced by a 3′-amino group. NPs exhibit both a high affinity towards a complementary RNA strand and nuclease resistance [40]. Their potency as AS molecules has already been demonstrated *in vivo*, where a phosphoroamidate ON was used to specifically down-regulate the expression of the *c-myc* gene [41]. As a consequence, severe combined immunodeficiency mice that were injected with myeloid leukemia cells had a reduced peripheral blood leukemic load. Animals treated with the AS agent had significantly prolonged survival compared to those treated with mismatch ONs. Moreover, the phosphoroamidates were found to be superior for the treatment of leukemia compared to phosphorothioate oligodeoxynucleotides. The sequence specificity of phosphoroamidate-mediated antisense effects by steric blocking of translation initiation could be demonstrated in cell culture, and *in vivo* with a system in which the target sequence was present just upstream of the firefly luciferase initiation codon [42]. Because phosphoroamidates do not induce RNase H cleavage of the target RNA, they might prove useful for special applications, where RNA integrity needs to be maintained, like modulation of splicing.

2′-Deoxy-2′-fluoro-β-D-arabino nucleic acid (FANA). ONs made of arabino nucleic acid, the 2′ epimer of RNA, or the corresponding 2′-deoxy-2′-fluoro-β-D-arabino nucleic acid analogue (FANA) were the first uniformly sugar-modified AS-ONs reported to induce RNase H cleavage of a bound RNA molecule [43]. The circular dichroic spectrum of a FANA·RNA duplex closely resembled that of the corresponding DNA·RNA hybrid, indicating similar helical conformations. The fluoro substituent is thought to project into the major groove of the helix, where it should not interfere with RNase H. Full RNase H activation by phosphorothioate–FANA, however, was only achieved with chimeric ONs containing deoxyribonucleotides in the center, but the DNA stretch needed for high enzyme activity was shorter than in 2′-*O*-methyl gapmers [44]. The chimeric FANA·DNA ONs were highly potent in cell culture with a 30-fold lower IC_{50} than the corresponding phosphorothioate DNA ON.

Locked nucleic acid (LNA). One of the most promising candidates of chemically modified nucleotides developed in the last few years is locked nucleic acid (LNA), a ribonucleotide containing a methylene bridge that connects the 2′-oxygen of the ribose with the 4′-carbon [reviewed in 36,45,46]. ONs containing LNA were first synthesized in the Wengel [47,48] and Imanishi laboratories [49] and are commercially available from Proligo (Paris, France and Boulder, CO, USA).

Introduction of LNA into a DNA ON induces a conformational change of the DNA·RNA duplex towards the A-type helix [50] and therefore prevents RNase H cleavage of the target RNA. If degradation of the mRNA is intended, a chimeric DNA·LNA gapmer that contains a stretch of 7–8 DNA monomers in the center to induce RNase H activity should be used [23]. Chimeric 2′-*O*-methyl–LNA ONs that do not activate RNase H could, however, be used as steric blocks to inhibit intracellular HIV-1 Tat-dependent *trans* activation and hence suppress gene expression [51]. LNAs and LNA·DNA chimeras efficiently inhibited gene expression when targeted to a variety of regions (5′ untranslated region, region of the start codon or coding region) within the luciferase mRNA [52].

Chimeric DNA·LNA ONs reveal an enhanced stability against nucleolytic degradation [23,53] and an extraordinarily high target affinity. An increase of the melting temperature of up to 9.6 °C per LNA introduced into an ON has been reported [50]. This enhanced affinity towards the target RNA accelerates RNase H cleavage [23] and leads to a much higher potency of chimeric DNA·LNA ONs in suppressing gene expression in cell culture, compared to phosphorothioate DNAs or 2′-*O*-methyl modified gapmers [A. Grünweller, E. Wyszko, V. A. Erdmann and J. Kurreck, unpublished results]. Whether the high target affinity of LNAs results in a reduced sequence specificity will need to be investigated. If unspecific side-effects of LNA

ONs are observed, their length would have to be decreased to find an optimum for target affinity and specificity.

AS-ONs containing LNA were also directed against human telomerase, which is an excellent antisense target that is expressed in tumor cells but not in adjacent normal tissue. Telomerase is a ribonucleoprotein with an RNA component that hybridizes to the telomere and should therefore be accessible for AS-ONs. As RNA degradation is not necessary to block the enzyme's catalytic site, ONs unable to recruit RNase H should be suitable inhibitors of telomerase function. A comparative study revealed that LNAs have a significantly higher potential to inhibit human telomerase than PNAs [54]. Due to their high affinity for their complementary sequence, LNA ONs as short as eight nucleotides long were efficient inhibitors in cell extracts.

In addition to target affinity, improved cellular uptake of ONs consisting of 2′-*O*-methyl RNA and LNA, compared to an all 2′-*O*-methyl RNA oligomer, was suggested to account for high antisense potency of LNA [51]. In the first *in vivo* study reported for an LNA, an efficient knock-down of the rat delta opioid receptor was achieved in the absence of any detectable toxic reactions in rat brain [53]. Subsequently, full LNA ONs were successfully used *in vivo* to block the translation of the large subunit of RNA polymerase II [55]. These ONs inhibited tumor growth in a xenograft model with an effective concentration that was five times lower than was found previously for the corresponding phosphorothioate DNA. Again, the LNA ONs appeared to be nontoxic in the optimal dosage. Therefore, full LNA and chimeric DNA·LNA ONs seem to offer an attractive set of properties, such as stability against nucleolytic degradation, high target affinity, potent biological activity and apparent lack of acute toxicity.

Morpholino oligonucleotides (MF). Morpholino ONs are nonionic DNA analogs, in which the ribose is replaced by a morpholino moiety and phosphoroamidate intersubunit linkages are used instead of phosphodiester bonds. They are commercially available from Gene Tools LLC (Corvallis, OR, USA). Recently, the success and limitations of their usage have been reviewed comprehensively, with particular focus on developmental biology [56] as most published work on morpholino compounds has been carried out using zebrafish embryos. An entire issue of *Genesis* (volume 30, issue 3, 2001) has been devoted to the study of gene function using this technique.

MFs do not activate RNase H and, if inhibition of gene expression is desired, they should therefore be targeted to the 5′ untranslated region or to the first 25 bases downstream of the start codon to block translation by preventing ribosomes from binding. Because their backbone is uncharged, MFs are unlikely to form unwanted interactions with nucleic acid-binding proteins. Their target affinity is similar to that of isosequential DNA ONs, but lower than the strength of RNA binding achieved with many of the other modifications described in this section.

Effective gene knockdown in all cells of zebrafish embryos was achieved with MFs against GFP in a ubiquitous GFP transgene [57]. In this study, equivalents of known genetic mutants as well as models for human diseases were developed and new gene functions were determined by the use of MFs. A potential therapeutic application was reported for MFs that corrected aberrant splicing of mutant β-globin precursor mRNA [58]. Treatment of erythroid progenitors from peripheral blood of thalassemic patients with ONs antisense to aberrant splice sites restored correct splicing and increased the hemoglobin A synthesis. Due to the limited cellular uptake of MFs, however, these experiments required high ON concentrations and mechanical disturbance of the cell membrane. Another relevant question that has to be answered is the reason for unspecific side-effects that have been observed in several studies (summarized in [56]).

Cyclohexene nucleic acids (CeNA). Replacement of the five-membered furanose ring by a six-membered ring is the basis for cyclohexene nucleic acids (CeNAs), which are characterized by a high degree of conformational rigidity of the oligomers. They form stable duplexes with complementary DNA or RNA and protect ONs against nucleolytic degradation [59]. In addition, CeNA·RNA hybrids have been reported to activate RNase H, albeit with a 600-fold lower k_{cat} compared to a DNA·RNA duplex [60]. Therefore, the design of ONs with CeNA has a long way to go in order to obtain highly efficient AS agents.

Tricyclo-DNA (tcDNA). Tricyclo-DNA (tcDNA) is another nucleotide with enhanced binding to complementary sequences, which was first synthesized by Leumann and coworkers [61,62]. As with most of the newly developed DNA and RNA analogs, tcDNA does not activate RNase H cleavage of the target mRNA. It was, however, successfully used to correct aberrant splicing of a mutated β-globin mRNA with a 100-fold enhanced efficiency relative to an isosequential 2′-*O*-methyl-phosphorothioate RNA [63].

In summary, a great number of modified building blocks for ONs have been developed during the last few years. Although not all of them could be discussed in the present review, general features have been shown for some promising examples. Most of the newly synthesized nucleotides reveal enhanced resistance against nucleolytic degradation in biological fluids and stabilize the duplex between the AS-ON and the mRNA. A major inherent disadvantage of nucleotides with modifications in the ribose moiety is their inability to activate efficient RNase H cleavage of the target RNA. As a consequence, gapmers with a stretch of unmodified or phosphorothioate DNA monomers in the center of the ON are widely used. Several of the third generation nucleotides have already been used successfully *in vivo*, and a high antisense potency combined with low toxicity has been observed. Therefore, one might expect that recent advances in nucleotide chemistry will soon lead to significant improvements of the antisense technology for target validation and therapeutic purposes.

Cellular uptake of antisense-oligonucleotides

Despite the encouraging prospects of nucleotide chemistry discussed in the previous section, an important hurdle that has to be overcome for successful antisense applications is the cellular uptake of the molecules. In cultured cells, internalization of naked DNA is usually inefficient, due to the charged ONs having to cross a hydrophobic cell

membrane. A number of methods have therefore been developed for *in vitro* and *in vivo* delivery of ONs (reviewed in [64,65]). By far the most commonly and successfully used delivery systems are liposomes and charged lipids, which can either encapsulate nucleic acids within their aqueous center or form lipid–nucleic acid complexes as a result of opposing charges. These complexes are usually internalized by endocytosis. For efficient release of the ONs from the endosomal compartment, many transfection reagents contain helper lipids that disrupt the endosomal membrane and help to set the ONs free.

A number of macromolar delivery systems have been developed recently that mediate a highly efficient cellular uptake and protect the bound ONs against degradation in biological fluids. Examples of these new agents are dendrimers with highly branched three dimensional structures, biodegradable polymers and ON-binding nanoparticles. In addition, pluoronic gel as a depot reservoir can be used to deliver ONs over a prolonged period [66]. It has been used *in vivo* successfully for the delivery of DNA enzymes (see below), which inhibited neointima formation after balloon injury to the rat carotid wall [67,68].

Further polymers for the delivery of AS-ONs consist of amino acids or sugars. Evidence has been provided, however, that the structural properties of a peptide conjugated to an ON do not significantly alter its ability to cross mammalian plasma membranes [69]. Therefore, aspects other than improved translocation across the membrane are likely to be responsible for enhanced biological activity of peptide–oligonucleotide derivatives. Further details about the newly developed delivery systems and perspectives for their wider use are given in the reviews mentioned above [64,65].

Another strategy for effective targeting of AS-ONs to specific tissues or organs is receptor-mediated endocytosis. For this purpose, ONs are conjugated to antibodies or ligands that are specifically recognized by a certain receptor, which mediates their uptake into target cells. For example, coupling of a radioactively labeled PNA to a transferrin receptor monoclonal antibody made the antisense agent transportable through the blood–brain barrier [70].

Interestingly, efficient cellular uptake of ONs *in vivo* has even been achieved without the use of any delivery system. In a recently published study it was demonstrated that fluorescently labeled AS-ONs were taken up by dorsal root ganglion neurons after intrathecal injection in the absence of any transfection agent [71]. The ONs specifically knocked down the expression of the peripheral tetrodoxin-resistant sodium channel NaV1.8 and reversed neuropathic pain induced by spinal nerve injury. Internalization into target cells *in vivo* has also been achieved for free ribozymes (see below). Despite these successful applications of free antisense molecules, higher levels of cellular uptake can usually be achieved by the use of transfection agents. Therefore, the development of delivery systems that mediate efficient cellular uptake and sustained release of the drugs remains one of the major challenges in the antisense field.

Clinical trials

After pharmacokinetic studies had shown that phosphorothioate oligodeoxynucleotides are well absorbed from parenteral sites and distribute broadly to organs and peripheral tissues [24] (with the exception that they do not cross the blood–brain barrier in the absence of special delivery systems) several companies initiated clinical trials in the early 1990s. As can be seen from the summary of ongoing clinical studies given in Table 1, the most intensively studied AS-ONs are phosphorothioate DNA ONs, but second and third generation ONs have meanwhile proceeded to Phase II trials. The list also demonstrates the

Table 1. Antisense-oligonucleotides approved or in clinical trials (compilation based on 16,37,81 and company's information).

Product	Company	Target	Disease	Chemistry	Status
Vitravene (Fomivirsen)	ISIS Pharmaceuticals	CMV IE2	CMV retinitis	PS DNA	Approved
Affinitac (ISIS 3521)	ISIS	PKC-α	Cancer	PS DNA	Phase III
Genasense	Genta	Bcl2	Cancer	PS DNA	Phase III
Alicaforsen (ISIS 2302)	ISIS	ICAM-1	Psoriasis, Crohn's disease, Ulcerative colitis	PS DNA	Phase II/III
ISIS 14803	ISIS	Antiviral	Hepatitis C	PS DNA	Phase II
ISIS 2503	ISIS	H-ras	Cancer	PS DNA	Phase II
MG98	Methylgene	DNA methyl transferase	Solid tumors	PS DNA	Phase II
EPI-2010	EpiGenesis Pharmaceuticals	Adenosine A1 receptor	Asthma	PS DNA	Phase II
GTI 2040	Lorus Therapeutics	Ribonucleotide reductase (R2)	Cancer	PS DNA	Phase II
ISIS 104838	ISIS	TNF α	Rheumatoid Arthritis, Psoriasis	2nd generation	Phase II
Avi4126	AVI BioPharma	c-myc	Restenosis, cancer, Polycystic kidney disease	3rd generation	Phase I/II
Gem231	Hybridon	PKA RIα	Solid tumors	2nd generation	Phase I/II
Gem92	Hybridon	HIV gag	AIDS	2nd generation	Phase I
GTI 2051	Lorus Therapeutics	Ribonucleotide reductase (R1)	Cancer	PS DNA	Phase I
Avi4557	AVI BioPharma	CYP3A4	Metabolic redirection of approved drugs	3rd generation	Phase I

almost universal applicability of antisense strategies to treat a broad range of diseases including viral infections, cancer and inflammatory diseases.

In 1998, the first (and to date only) antisense drug Vitravene (Fomivirsen), was approved by the US Food and Drug Administration [72]. The phosphorothioate DNA is intravitreally injected to treat cytomegalovirus-induced retinitis in patients with AIDS. Approval of Vitravene was a milestone for companies involved in the antisense field. The drug meets an important need for affected patients, but its application is rare so that it generated only about $157 000 in sales for ISIS Pharmaceuticals (Carlsbad, CA, USA) and Novartis (Basel, Switzerland) in 2001 [16].

Three antisense phosphorothioate oligodeoxynucleotides are currently being investigated in Phase III trials. Affinitac (ISIS 3521) is targeted against the protein kinase C-alpha (PKC-α) for the treatment of nonsmall-cell lung cancer. The successful trial caught the attention of big pharmaceutical companies and led to a $200 million deal between Eli Lilly (Indianapolis, IN, USA) and ISIS Pharmaceuticals [73]. This deal marked the recovery from a serious setback for ISIS in 1999, when Alicaforsen (ISIS 2302) failed to show significant efficacy in a Phase III study, where it was tested for treatment of Crohn's disease [74]. This AS-ON is now being investigated in a restructured Phase III trial. Genta (Berkeley Hights, NJ, USA) is developing the anticancer drug Genasense, which attacks the apoptosis inhibitor Bcl2 and shows antitumor responses in patients with malignant melanomas [75].

Further antiviral or anticancer phosphorothioate DNAs are being investigated in Phase I or II trials. Most of the antisense molecules currently being tested are intravenously or subcutaneously injected, but EpiGenesis Pharmaceuticals (Cranbury, NJ, USA) developed a 'respirable antisense-oligonucleotide' (RASON) targeting the adenosine A_1 receptor to treat asthma [76]. It has a duration of effect of approximately one week, giving it the potential to be the first once-per-week treatment for this disease.

Recently, results of a pilot study for the treatment of chronic myelogenous leukemia patients were presented [77]. Marrow cells were purged *ex vivo* with a phosphorothioate oligodeoxynucleotide against the short-lived *c-myb* proto-oncogene. The treatment led to major cytogenetic remissions in six of an evaluable 14 patients. An infusion trial with the c-myb AS-ONs in patients with refractory leukemia of all types has been approved and is expected be started soon (A. M. Gewirtz, Division of Haematology/Oncology, University of Pennsylvania School of Medicine, Philadelphia, USA, personal communication).

Furthermore, several second generation ONs have reached the stage of clinical trials. ISIS 104838 against tumor necrosis factor α (TNFα) is being tested for the treatment of inflammatory diseases such as rheumatoid arthritis and psoriasis, and Hybridon (Cambridge, MA, USA) uses second generation drug candidates to treat cancer and HIV infections. Mixed backbone oligonucleotides consisting of phosphorothioate internucleotide linkages and four 2′-*O*-methyl RNA nucleotides at both ends were shown to have antitumor activity in mice after oral administration [78].

Mixed backbone oligonucleotides usually contain phosphorothioate internucleotide linkages even between the 2′-*O*-methyl nucleotides. Thus, the number of phosphorothioates is not decreased compared to an entirely phosphorothioate DNA ON, but for reasons unknown to date their toxicity is significantly reduced. Regardless of this open question, AS-ONs containing second generation modifications combine several advantageous properties, including higher *in vivo* stability, better pharmacological and toxicological profiles and the opportunity for oral administration.

Third generation AS-ONs with a morpholino-type backbone are being tested in Phase I and II clinical trials by Avi BioPharma (Portland, OR, USA). Avi4126 targets the oncogene *c-myc* and is used to treat restenosis, polycystic kidney disease and solid tumors [79]. A second MF-ON against cytochrome P450 (CYP3A4) is being designed for metabolic redirection of approved drugs. An N3′-P5′-thiophosphoroamidate that efficiently inhibited telomerase activity in spontaneously immortalized human breast epithelial cells [80] will soon be moved to clinical trials by Geron (Menlo Park, CA; S. Gryaznov, personal communication).

Although the AS molecules have been well-tolerated in most cases and some results were encouraging, no or only minor responses were achieved in several studies [81]. Taken together, an increasing number of AS-ONs have been investigated in different stages of clinical trials and a broad spectrum of diseases is addressed in these studies, but some questions remain to be answered. Solutions to major problems of serum-stability, bioavailability, tissue-targeting and cellular delivery urgently need to be found. Most of the antisense molecules used are still phosphorothioate oligodeoxynucleotides, but some second and third generation chemistry molecules are being tested and seem to provide favorable pharmacokinetic properties and the opportunity of oral administration.

Ribozymes

In the early 1980s, Cech and coworkers discovered the self-splicing activity of the group I intron of *Tetrahymena thermophilia* [82,83] and coined the term 'ribozymes' to describe these RNA enzymes. Shortly thereafter, Altman and colleagues discovered the active role of the RNA component of RNase P in the process of tRNA maturation [84]. This was the first characterization of a true RNA enzyme that catalyses the reaction of a free substrate, i.e. possesses catalytic activity *in trans*. A variety of ribozymes, catalyzing intramolecular splicing or cleavage reactions, have subsequently been found in lower eukaryotes, viruses and some bacteria. The different types of ribozymes and their mechanisms of action have been described comprehensively [85–89] and the present review will therefore focus on the stabilization and medical application of the hammerhead ribozyme, which has been studied in great detail and is one of the most widely used catalytic RNA molecules.

The hammerhead ribozyme was isolated from viroid RNA and its dissection into enzyme and substrate strands [90,91] transformed this *cis*-cleaving molecule into a target-specific *trans*-cleaving enzyme with a great potential for applications in biological systems. This minimized hammerhead ribozyme is less than 40 nucleotides long and consists of two substrate binding arms and a catalytic domain (Fig. 5).

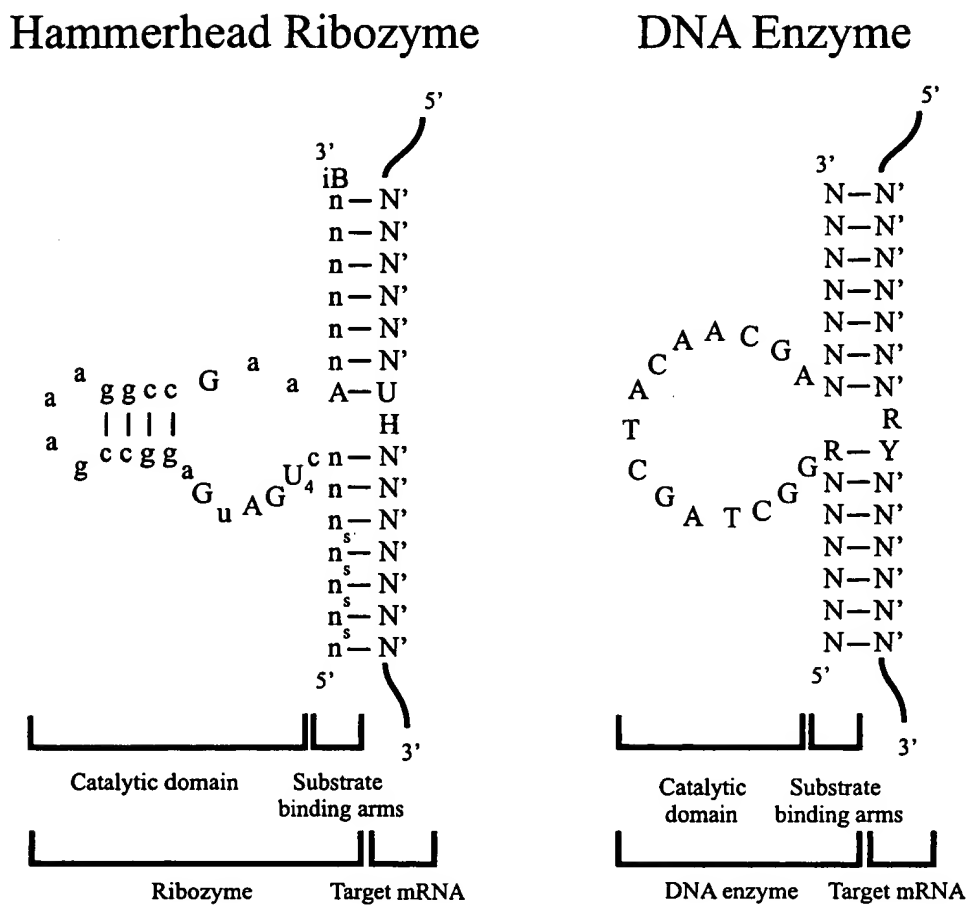

Fig. 5. Secondary structure models for the hammerhead ribozyme and the 10-23 DNA enzyme. A nuclease-resistant ribozyme according to Usman and Blatt [111] is shown. It consists of 2′-*O*-methyl RNA (lower case), five ribonucleotides (upper case), a 2′-C-allyluridin at position 4, four phosphorothioate linkages (s) and an inverted 3′-3′ deoxabasic sugar. The DNA enzyme shown consists entirely of DNA nucleotides; R is a purine, Y is a pyrimidine.

For the development of a therapeutic hammerhead ribozyme similar problems have to be solved as described for AS-ONs. Some steps, however, are more challenging due to the catalytic nature of ribozymes. Firstly, suitable target sites have to be identified, secondly the oligoribonucleotides have to be stabilized against nucleolytic degradation and thirdly the ribozymes have to be delivered into the target cells.

Hammerhead ribozymes are known to cleave any NUH triplets (where H is any nucleotide except guanosine) with AUC and GUC triplets being processed most efficiently. Triplets with a cytidine or an adenosine at the second position were reported to be cleavable by hammerhead ribozymes [92], although these reactions occurred at lower rates. Due to secondary and tertiary structures of the target mRNAs, not all sequences that are theoretically cleavable by hammerhead ribozymes are suitable for practical applications. Therefore, several assays have been developed to identify accessible target sites.

A good correlation was found for regions of the c-myb mRNA that were accessible to AS-ON binding in an RNase H assay and their susceptibility to cleavage by ribozymes *in vitro* [93]. Oligonucleotide scanning of the DNA methyltransferase mRNA in cell extracts had also been found to be predictive for ribozyme activity in cell extracts and inside cells [94].

Another approach for the identification of active ribozymes was based on the usage of libraries with randomized substrate recognition arms. The hammerhead ribozymes have either been transcribed from expression cassettes [95] or were chemically synthesized [96]. A highly sophisticated method was developed, in which a sequence-specific library of hammerhead ribozymes was generated by partial digestion of the target cDNA and subsequent introduction of the catalytic domain into the library [97].

For applications in cell culture or *in vivo*, ribozymes can either be transcribed from plasmids inside the target cells or they can be administered exogenously. The first approach requires the design of expression cassettes with an RNA polymerase III promoter and stem-loop structures that stabilize the ribozyme (reviewed in [98]). Some gene therapy-based trials have been performed to treat individuals infected with HIV (summarized in [99]). Because the use of chemically synthesized ribozymes proved to be more straightforward, this approach will be discussed in more detail below. Due to the fact that RNA is rapidly degraded in biological systems, presynthesized ribozymes have to be protected against nucleolytic attack before they can be used in cell culture or *in vivo*.

Stabilization of ribozymes is even more difficult than protection of AS-ONs, as the introduction of modified

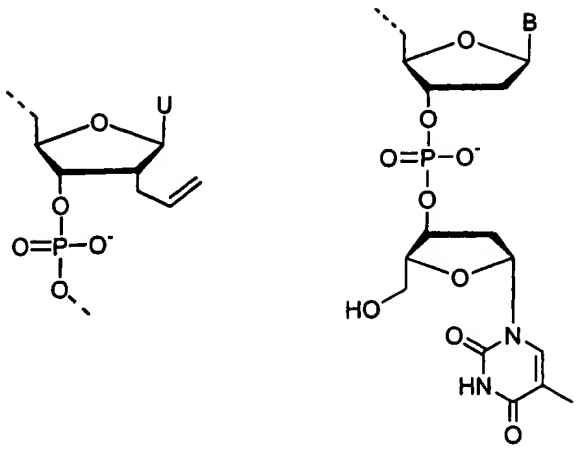

Fig. 6. Modified nucleotides used to stabilize ribozymes and DNA enzymes.

nucleotides very often leads to conformational changes that abolish catalytic activity. Based on a number of reports, in which sequence–function relationships in the hammerhead ribozyme were analyzed, a comprehensive study was performed using a great variety of modified nucleotides that led to an optimized design for a stabilized hammerhead ribozyme, which is almost as active as its unmodified parent [100]. The nuclease resistant ribozyme contains five unmodified ribonucleotides, a 2′-*C*-allyl uridine (Fig. 6) at position 4 and 2′-*O*-methyl RNA at all remaining positions. In addition, the 3′ end was protected by an inverted thymidine. The serum half-life of the stabilized ribozyme is increased to more than 10 days compared to a less than 1 min half-life of the unmodified RNA ribozyme. A slightly improved version of this ribozyme with four phosphorothioate bonds in one substrate recognition arm and an inverted 3′-3′ deoxyabasic sugar led to the design presented in Fig. 5 that is now used for clinical trials (see below).

The development of *in vitro* selection techniques using combinatorial libraries opened the road to generate ribozymes with advantageous properties such as the accessibility of new target sites [101], high activity under physiological Mg^{2+} concentrations [102] and enhanced biostability (reviewed in [103]). A highly active ribozyme against a *K-ras* target sequence could be selected in the presence of 2′-fluoro and 2′-amino modified ribonucleotides [104]. The optimized ribozyme that was named Zinzyme has a relatively high catalytic activity at 1 mM Mg^{2+} and cleaves a new Y-G-H (where Y is C or U, and H is A, C or U) target sequence. Two unmodified guanosines and two 2′-amino nucleotides are essential for cleavage activity, 2′-*O*-methyl RNA could be introduced at all other positions. The arms are further stabilized by phosphorothioate linkages and an inverted 3′-3′ deoxyabasic sugar as described above. The Zinzyme has a half-life of > 100 h in human serum.

Ribonucleotides, which are highly susceptible to nucleases, could be avoided entirely by the selection of an RNA-cleaving DNA enzyme [105]. The most prominent deoxyribozyme, named '10-23', consists of a catalytic core of 15 nucleotides and two substrate recognition arms of 6–12 nucleotides on either arm (Fig. 5). It is highly sequence-specific and can cleave any junction between a purine and a pyrimidine (review [106]). A comparative study of hammerhead ribozymes and DNA enzymes targeting the same cleavage sites of a long mRNA revealed that no general conclusions can be drawn as to whether the hammerhead ribozyme or the DNA enzyme is more efficient, but the most active cleaver found in the study was a 10-23 DNA enzyme [11].

Addition of an inverted nucleotide at the 3′ end enhanced serum stability of the 10-23 DNA enzyme 10-fold (the half-life of the modified DNA enzyme was 20 h compared to less than 2 h for the unmodified deoxyribozyme) [107]. DNA enzymes with a 3′-3′ inverted thymidine have also been used in the first *in vivo* application and inhibited neointima formation after balloon injury [67]. Sequence requirements in the catalytic core of the 10-23 DNA enzyme were analyzed and revealed a higher degree of conservation at the borders than in between [108]. A DNA enzyme with optimized substrate recognition arms and a partially protected catalytic domain possessed not only increased nuclease resistance but also enhanced catalytic activity [S. Schubert and J. Kurreck, unpublished results].

For transfection of eukaryotic cells with ribozymes, similar strategies can be used as have been described above for AS-ONs. Again, cationic lipids are most commonly used for cell culture experiments, but successful application of ribozymes in an animal model was demonstrated in the absence of any delivery system [109]. Chemically stabilized ribozymes were taken up by cells in the synovial lining after intra-articular administration and reduced the interleukin 1α-induced stromelysin mRNA. Higher transfection efficiencies can, however, usually be achieved with delivery systems. In addition, it could be shown that low molecular mass poly(ethylenimine) not only mediates highly efficient cellular uptake of ribozymes but also stabilizes RNA against nucleolytic degradation [110]. Poly(ethylenimine)-complexed ribozymes consisting of unmodified RNA were stable in cell culture and *in vivo*, and reduced tumor growth in a mouse xenograft model.

One of the leading companies in the field, Ribozyme Pharmaceuticals (Boulder, CO, USA), performs clinical trials (Table 2) using stabilized hammerhead ribozymes [111] as well as Zinzymes. ANGIOZYME is a stabilized hammerhead ribozyme that is targeted against the vascular endothelial growth factor (VEGF) receptor. It is designed to reduce tumor growth by inhibition of the formation of new blood vessels (angiogenesis). An additional benefit is expected from the combination of ANGIOZYME with chemotherapy in the treatment of metastatic colorectal

Table 2. Chemically synthesized ribozymes of Ribozyme Pharmaceuticals in ongoing clinical trials (P. Pavco, Ribozyme Pharmaceuticals, personal communication).

Product	Target	Disease	Status
ANGIOZYME	VEGF-receptor 1	Metastatic colorectal cancer	Phase II
HERZYME	HER-2	Cancer	Phase I

cancer. For further details about the current status of ribozymes as therapeutic agents for cancer and problems in progressing from cell culture studies to *in vivo* models and clinical trials, see Wright and Kearney [112].

HEPTAZYME is another modified hammerhead ribozyme that cleaves the internal ribosome entry site of the Hepatitis C virus. The ribozyme was demonstrated to inhibit viral replication up to 90% in cell culture [113]. HEPTAZYME was tested in a Phase II study, but is no longer in a clinical trial (P. Pavco, Ribozyme Pharmaceuticals, personal communication). HERZYME is a Zinzyme that is targeted against the human epidermal growth factor-2 (HER2), which is overexpressed in certain breast and ovarian cancers. This ribozyme is being tested in a Phase I trial (P. Pavco, Ribozyme Pharmaceuticals, personal communication) to gain information about the safety and the adequateness of the pharmacokinetics of HERZYME.

RNA interference

Only recently, research in the antisense field increased in impact by the discovery of RNA interference (RNAi). This naturally occurring phenomenon as a potent sequence-specific mechanism for post-transcriptional gene silencing was first described for the nematode worm *Caenorhabditis elegans* [114]. Due to the advances made in the RNAi field during the last two years, numerous reviews have been published only recently [115–117]. RNA interference is initiated by long double-stranded RNA molecules, which

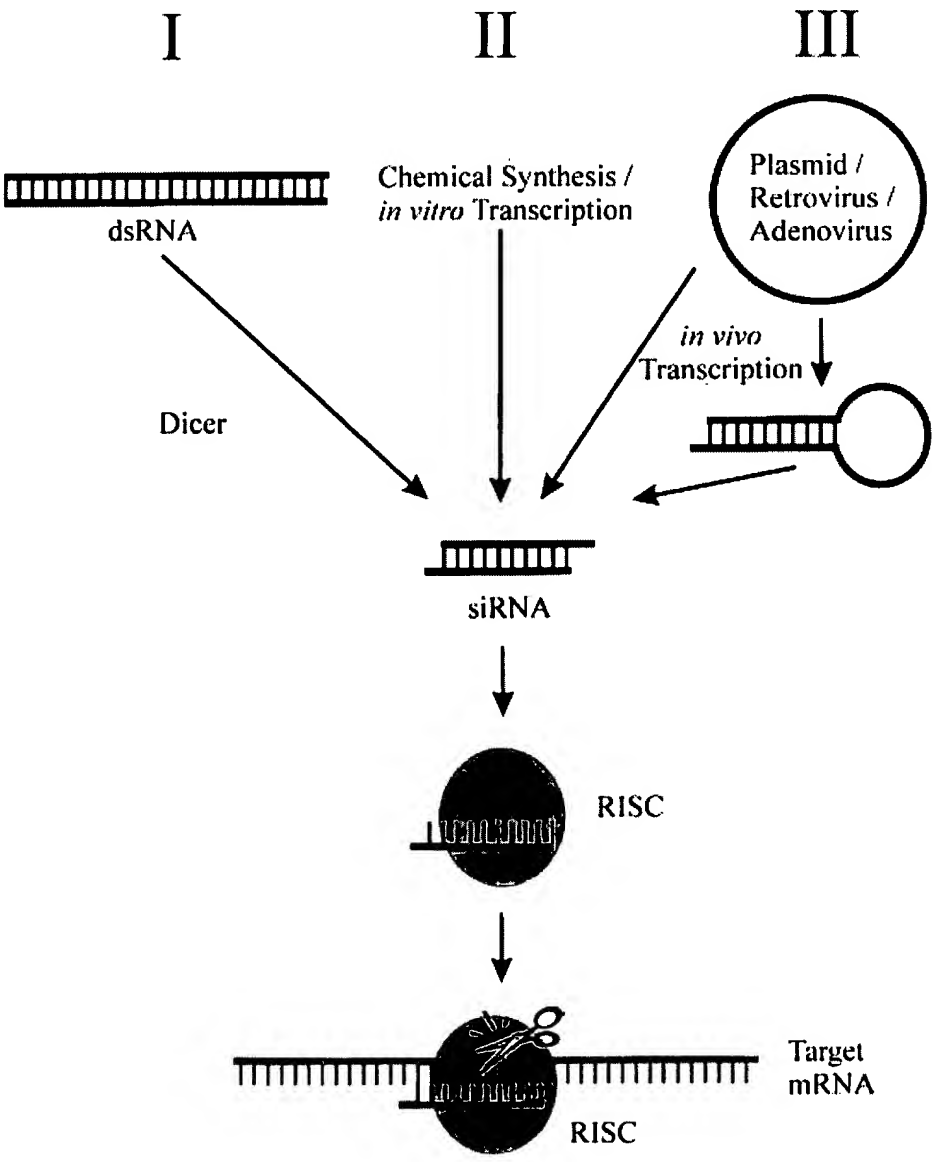

Fig. 7. Gene silencing by RNA interference (RNAi). RNAi is triggered by siRNAs, which can by generated in three ways. (I) Long double-stranded RNA molecules are processed into siRNA by the Dicer enzyme; (II) chemically synthesized or *in vitro* transcribed siRNA duplexes can be transfected into cells; (III) the siRNA molecules can be generated *in vivo* from plasmids, retroviral vectors or adenoviruses. The siRNA is incorporated into the RISC and guides a nuclease to the target RNA.

are processed into 21–23 nucleotides long RNAs by the Dicer enzyme (Fig. 7). This RNase III protein is thought to act as a dimer that cleaves both strands of dsRNAs and leaves two-nucleotide, 3′ overhanging ends. These small interfering RNAs (siRNAs) are then incorporated into the RNA-induced silencing complex (RISC), a protein·RNA complex, and guide a nuclease, which degrades the target RNA.

This conserved biochemical mechanism could be used to study gene functions in a variety of model organisms, but its application to mammalian cells was hampered by the fact that long double-stranded RNA molecules induce an interferon response. It was therefore a revolutionary breakthrough, when Tuschl and coworkers could show that 21 nucleotide-long siRNA duplexes with 3′ overhangs can specifically suppress gene expression in mammalian cells [2]. This finding triggered an enormous number of studies using RNAi in mammalian cells, as it is thought to provide a significantly higher potency compared to traditional antisense approaches.

Interestingly, not only short double-stranded RNA molecules but also short hairpin RNAs (shRNAs), i.e. fold-back stem-loop structures that give rise to siRNA after intracellular processing, can induce RNA interference [118,119]. This opened up the possibility of constructing vectors expressing the interfering RNA for long-term silencing of gene expression in mammalian cells (summarized in [117,120]). Short hairpin RNA was transcribed using RNA polymerase III promotors that normally control the transcription of either the small nuclear RNA U6 [118,119,121,122] or the H1 RNA component of RNase P [123]. Alternatively, two short RNA molecules were transcribed separately using two U6 promotors [118,124,125]. Vector-mediated expression of siRNA allows the analysis of loss-of-function phenotypes that develop over a longer period of time. In stably transfected cells, silencing was observed even after two months [123].

An alternative approach to prolong siRNA-mediated inhibition of gene expression is the introduction of modified nucleotides into chemically synthesized RNA, despite the fact that even unmodified short double-stranded RNA revealed an unexpectedly high stability in cell culture and *in vivo*. For certain applications, however, further enhancement of the siRNA stability might be desirable. Therefore, modified nucleotides were introduced to the ends of both strands [126]. A siRNA with two 2′-*O*-methyl RNA nucleotides at the 5′ end and four methylated monomers at the 3′ end was as active as its unmodified counterpart and led to a prolonged silencing effect in cell culture. Extension of the methylated stretch of nucleotides as well as the introduction of nucleotides with a bulky 2′-allyl substituent resulted in decreased siRNA activity.

For the first *in vivo* studies of RNA interference in mammals the siRNA or a plasmid coding for shRNA was delivered using rapid injection of a large volume of physiological solution into the mouse tail vein [127,128]. Expression of reporter genes that were either encoded on cotransfected plasmids or in transgenic mouse strains could efficiently be inhibited in most of the organs. In addition, the *Fas* gene has been targeted as an endogenous, therapeutically relevant target for liver injury [129]. After siRNA injection, the Fas mRNA and protein levels were reduced in mouse hepatocytes for 10 days. Silencing Fas protected mice from fulminant hepatitis induced by injection of agonistic Fas-specific antibody; 82% of mice treated with siRNA survived the 10 days of observation, whereas all control animals died within three days.

The high-pressure delivery technique used in the studies described above is, however, a rather harsh method that might influence results and cannot be used for therapeutic applications. Therefore, methods known from standard gene therapy have been adapted for RNA interference. A retroviral vector was used to deliver siRNA that inhibited the carcinogenic *K-ras* allele in human pancreatic tumor cells [130]. Down-regulation of *K-ras* expression in carcinoma cells abolished their ability to form tumors after subcutaneous injection into athymic nude mice. This study also demonstrated the high specificity of siRNA, as only the carcinogenic *K-ras* but not the wild type *K-ras* allele, which differs by only one base pair, was silenced. Furthermore, GFP expression could be suppressed in the brain of transgenic mice after injection of adenovirus vectors expressing siRNA into the striatal region [131]. Activity of endogenous β-glucoronidase could be decreased by injecting recombinant adenoviruses into the mouse tail vein. Interestingly, an RNA polymerase II expression cassette with a CMV promoter and a minimal poly(A) was used for the latter experiments, opening the door to design tissue-specific or inducible siRNA vectors.

Taken together, first promising *in vivo* experiments with siRNA have already been performed and further therapeutically important genes are expected to be targeted soon. No toxic reactions after siRNA application have been observed in the studies performed to date, but great care has to be taken to rule out severe side-effects of long-term induction of RNAi before trials can be started to treat human diseases. Because silencing of gene expression by siRNAs is similar to traditional antisense technology, researchers will be able to benefit from the lessons learned for more than a decade such as the requirement to use proper controls to proof a specific knock-down of gene expression and a careful analysis of possible unspecific effects mediated by the immune system.

Summary

After a long period of ups and downs, antisense technologies have gained increasing attention in recent years. Major improvements have been achieved by the development of modified nucleotides that provide high target affinity, enhanced biostability and low toxicity. As most of the new DNA analogs do not induce RNase H cleavage, the design of antisense-oligonucleotides has to be adjusted depending on whether the target mRNA has to remain intact, e.g. for alteration of splicing, or should be degraded (gapmer technology). Stable ribozymes with high catalytic activity were obtained by systematically modifying naturally occurring ribozymes or by *in vitro* selection techniques. Several antisense-oligonucleotides and ribozymes are currently being investigated in clinical trials and one antisense drug was approved in 1998. A major breakthrough was the discovery that short double-stranded RNA molecules can be used to silence gene expression specifically in mammalian

cells. This method has a significantly higher efficiency compared to traditional antisense approaches and some promising *in vivo* data have already been presented. Therefore, antisense technologies can be expected to be widely used for studies of genes with unknown function, for target validation in drug development and finally, of course, for therapeutic purpose.

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Antisense molecules and furanose conformations—is it really that simple?

Lisbet Kværnø†[a] and Jesper Wengel*[b]

[a] *Center for Synthetic Bioorganic Chemistry, Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark*
[b] *Department of Chemistry, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark. E-mail: jwe@chem.sdu.dk; Fax: +45 66 15 87 80*

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Selected nucleic acid mimics are discussed in the context of the antisense therapeutic strategy with focus on furanose conformation, RNA-binding affinity, and activation of the RNA-cleaving enzyme RNase H.

Introduction

In order to inhibit gene expression by targeting RNA with short antisense oligonucleotides, multiple chemically modified nucleoside and oligonucleotide analogues have been synthesized and evaluated during the last 15 years.[1,2] Selected examples of modified nucleic acids are described here with the emphasis on their hybridization properties and capability to activate RNase H, an RNA-degrading enzyme, in relation to their conformational and structural characteristics. The focus is on important recent developments and their prospects towards the realization of the antisense therapeutic strategy.

The antisense strategy

The carriers of all genetic information in living organisms are 2′-deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), polymers consisting of repetitive units of nucleotide monomers. Each nucleotide contains a phosphate group, a carbohydrate moiety and a nucleobase (Fig. 1). The natural nucleobases in

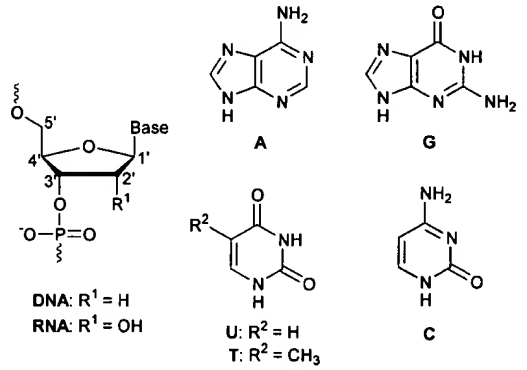

Fig. 1 Structures of a nucleotide monomer (left) and the nucleobases adenine (A), guanine (G), uracil (U), thymine (T), and cytosine (C).

DNA (R^1 = H) are A, G, T and C while U replaces T in RNA (R^1 = OH). Two complementary polymeric or oligomeric single strands hybridize in an anti-parallel fashion forming a right-handed duplex with the specific Watson–Crick base-pairing of G to C and A to T or U.

As depicted schematically in Fig. 2, the genetic information is transcribed from double-stranded DNA, located in the cell

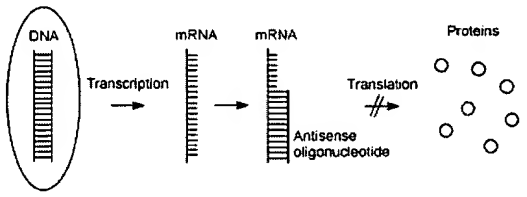

Fig. 2 Schematic overview of the antisense strategy.

nucleus, to single-stranded messenger-RNA (mRNA) which functions as a carrier of genetic information from the cell nucleus to the ribosomes in the cytoplasm. The principle of the antisense strategy is to target mRNA by duplex formation using a short (10–20 nucleotides long) antisense oligonucleotide, thereby preventing the translation of the mRNA into proteins. In principle, any genetic sequence, and thus any disease with a genetic origin, should be subject to selective targeting by varying the nucleotide sequence of an antisense oligonucleotide approximately 18 nucleotides long. This makes the antisense strategy general and attractive compared with the individual development of traditional drugs acting at the protein level. An antisense oligonucleotide has to fulfil several functional requirements, *e.g.* high binding affinity toward the RNA binding strand, resistance towards nucleases, low toxicity, and efficient delivery to the desired target site, *in vivo*, rendering

Lisbet Kværnø (b. 1974) received her BSc and MSc degrees in 1998 and 2000, respectively, from The University of Copenhagen under the supervision of Professor Jesper Wengel. She is currently pursuing a PhD within the field of total synthesis of natural products in the group of Professor David Tanner, The Technical University of Denmark.

Jesper Wengel (b. 1963) obtained his PhD within the field of nucleoside chemistry in the group of Dr Erik B. Petersen, University of Southern Denmark, Odense (formerly Odense University) in 1991 and joined the faculty immediately afterwards as an assistant professor. In 1996, he was appointed as a full professor in bioorganic chemistry at The University of Copenhagen. He returned to The University of Southern Denmark, Odense, also as a full professor, in August 2000. His main research interests are the synthesis and properties of chemically modified nucleosides and oligonucleotides, and the use of branched and functionalized oligonucleotides within supramolecular chemistry.

† Current address: Department of Chemistry, The Technical University of Denmark, Building 201, DK-2800, Lyngby, Denmark.

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chemical modification of the natural nucleic acid structures needed.

Nucleic acid structure and furanose conformations

The pseudorotational circle[3] depicted in Fig. 3 describes the possible furanose conformations of the nucleotide monomers.

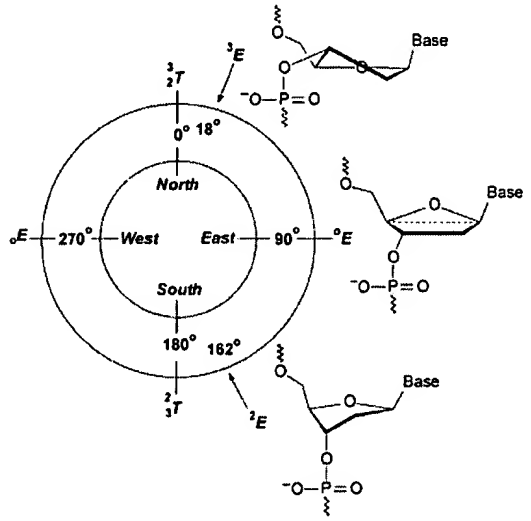

Fig. 3 The pseudorotation cycle showing the relation between the pseudorotation angle P (0–360°; calculated from the five torsional angles of the furanose ring[3]) and furanose ring conformations.

All distinct conformations (*i.e.* envelope, *E*, and twist, *T*, conformations) are separated by 18° with a superscript designating an atom above the plane described by the remaining three or four atoms of the furanose ring, and a subscript designating an atom below.

The two dominating furanose conformations for DNA and RNA generally give rise to two different duplex forms, as depicted in Fig. 4. Apart from having all furanose conformations in an *S*-type (*south*-type, C2′-*endo*, ^2E) conformation, the B-type helix seen in solution for DNA is characterized by 10 base-pairs almost perpendicular to the helix axis per helix turn, and a distinct difference in width of the major and the minor grooves.[4,5] In contrast, RNA adopts an A-type helix in which the furanose conformations are all *N*-type (*north*-type, C3′-*endo*, ^3E) with 11 base-pairs tilted 20° with respect to the helix axis per helix turn, and the minor and major grooves being almost equally wide.[4,5]

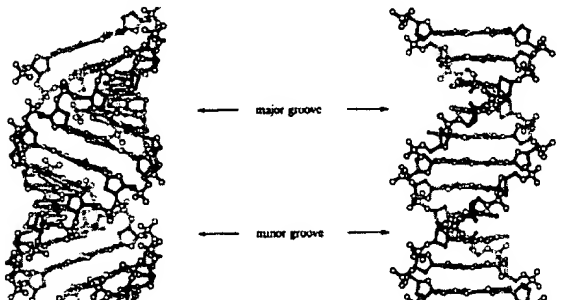

Fig. 4 Globular conformations of an A-type duplex (left) and a B-type duplex (right) generally seen for RNA:RNA and DNA:DNA duplexes, respectively.

Modes of action and conformational requirements of antisense molecules

An antisense oligonucleotide has basically two possible modes of action both depending on the duplex formation between the RNA target and the antisense oligonucleotide. One is a simple steric blocking of the mRNA, the other recognition of the RNA–antisense duplex as a substrate for the enzyme RNase H which cleaves the RNA strand of an RNA–DNA duplex. In the latter scenario, one antisense oligonucleotide is able to pacify multiple mRNA strands. A high binding affinity towards RNA is crucial, especially for the steric blocking approach. Conformational restriction of the single-stranded antisense oligonucleotides is believed to favour duplex formation entropically by diminishing the loss of conformational degrees of freedom upon duplex formation, and conformational restriction of the furanose rings of oligonucleotides has been successfully applied to the field in the recent years.[6–8] Studies of a large number of oligonucleotide analogues have been generalized to the hypothesis that oligonucleotides restricted into *N*-type furanose conformations, thus yielding A-type duplexes when hybridized to RNA, effect the highest duplex stabilities.[1,9]

However, no fully modified antisense oligonucleotide with restricted *N*-type furanose conformations has been reported to be able to activate RNase H. The RNA–DNA heteroduplexes being substrates of RNase H which has been reported to bind in the minor groove, adopt an intermediate duplex form with a minor groove width also intermediate between that of the A form and the B form.[10–14] The furanose conformations in the RNA strand are still *N*-type (3′-*endo*) while hybridization to the RNA strand causes the furanose conformations of the DNA strand to change from the typical *S*-type (C2′-*endo*) into *E*-type conformations (O4′-*endo* range, see Fig. 3).[10,11,14] Thus, the activation of RNase H proposedly requires antisense oligonucleotides with furanose rings able to adopt *E*-type (O4′-*endo*), or perhaps *S*-type (C2′-*endo*), conformations, and *not* the duplex-stabilizing *N*-type conformations.

An appealing way to circumvent the lack of RNase H activation is the introduction of chimeric structures consisting of high-affinity nucleotide modifications in the terminal regions of the antisense oligonucleotide around a 'gap' of RNase H-activating nucleotides in the central part. To activate the mammalian RNase H, a gap size of five[15] to seven[16] unmodified 2′-deoxynucleotides has been suggested.

Selected oligonucleotide analogues

A number of the more promising nucleic acid mimics for the development of successful antisense oligonucleotides are discussed in the following. Emphasis is given to analogues in which the furanose ring is restricted into a distinct conformation by either stereoelectronical or constitutional (sterical) means. Average duplex stabilities for hybridization towards complementary RNA targets are reported as the change in the melting temperature per modified nucleotide (°C per mod.) relative to the corresponding unmodified DNA–RNA reference duplex, the melting temperature being defined as the temperature at which half of the duplex is dissociated as measured by the associated hyperchromic shift at 260 nm.

Phosphorothioates

In 1998, the first antisense drug, Vitravene™ (ISIS-2922), was approved by the FDA for the treatment of cytomegalovirus retinitis in immunocompromised patients.[17] This antisense oligonucleotide consists of phosphorothioate (thiophosphate) nucleotides, in which one of the non-bridging phosphate oxygens is substituted by sulfur (**5**, Fig. 5) resulting in improved nuclease stability while the capability to activate RNase H remains intact. Phosphorothioates are, however, considered as

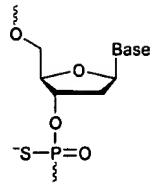

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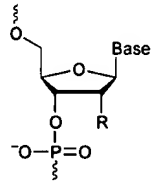
Fig. 5 Structure of phosphorothioates.

only 'first generation' antisense oligonucleotides due to less desirable features such as lowered duplex stabilities (approximately −0.8 °C per mod.), the existence of diastereomeric mixtures because of the chirality of the phosphorus atoms, and significant toxicity caused, *e.g.* by non-specific binding to proteins.[18]

Selected 2′-*O*-alkylated RNA derivatives

For 2′-*O*-alkylated RNA derivatives, the *gauche* effect between O2′ and O4′ (along the C1′–C2′ bond) induces a conformational shift towards an *N*-type (C3′-*endo*) furanose conformation resulting in A form duplexes as also seen for unmodified RNA. Provided that the 2′-*O*-alkyl group is not too sterically demanding (*i.e.* alkyls smaller than hexyl),[19] increased duplex stability is observed. A 2′-*O*-Me-RNA monomer (**6A**, Fig. 6)



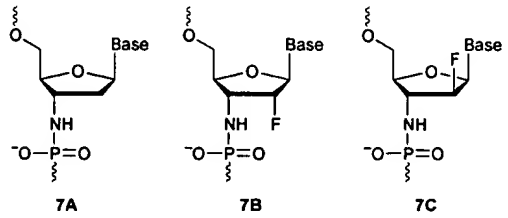
6A R = OCH_3
6B R = $OCH_2CH_2OCH_3$ (MOE)
6C R = $OCH_2CH_2CH_2NH_2$ (AP)

Fig. 6 Structures of selected 2′-*O*-alkylated RNA derivatives.

induces an increase in the melting temperature of *ca.* +0.5 to +1.0 °C per mod.,[20] while 2′-*O*-(methoxyethyl)- (MOE, **6B**) and 2′-*O*-aminopropyl- (AP, **6C**) monomers effect increases of *ca.* +1.0 to +1.5 °C per mod.[20,21] and *ca.* +1.0° C per mod.,[22] respectively. The nuclease stability of the 2′-*O*-Me derivatives is insufficient for antisense purposes,[9] which suggests the use of the more resistant MOE[21] or AP[22] modifications. Because of their preorganization into an *N*-type furanose conformation, none of the 2′-*O*-alkylated derivatives activate RNase H unless a gap-mer strategy is applied.[23]

N3′ → P5′-Phosphoramidates

Replacing the 3′-hydroxy group with an amino group introduces a non-chiral nuclease-resistant phosphoramidate internucleoside linkage. Such a modification (**7A**, Fig. 7) increases duplex



7A **7B** **7C**

Fig. 7 Structures of N3′ → P5′-phosphoramidates.

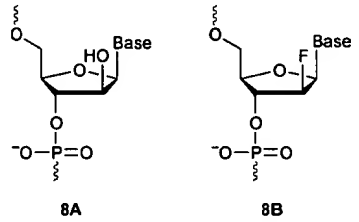
stabilities by +2.3 to +2.6 °C per mod.[24–26] which has been explained by the diminished *gauche* effect from the 3′-nitrogen atom preorganizing the furanose ring into an *N*-type (C3′-*endo*) conformation thus furnishing A-type duplexes with RNA targets as also shown by NMR[27,28] and X-ray studies.[29] Hybridized to RNA, fully modified phosphoramidates do not activate RNase H,[30] but this problem can be circumvented by the use of a gap-mer sequence with unmodified DNA monomers in the central gap[31] and also of a phosphoramidate mix-mer with alternating phosphoramidate and unmodified phosphordiester nucleotides.[32] The gap-mer phosphoramidates showed a lack of antisense activity which might be ascribed to the endonucleolytic degradation of the deoxynucleotide gap[9] while only fully modified phosphoramidates showed an RNase H-independent antisense activity.[31]

2′-Substitution with a *ribo*-configured fluorine atom (**7B**, *ribo*-configuration, Fig. 7) constrains the furanose ring entirely to a C3′-*endo* conformation due to the strong *gauche* effect from fluorine and induces increased duplex stabilities with as much as +4 to +5 °C per mod.[33] The RNA analogue of **7B** (a 2′-OH group instead of the fluorine atom) likewise afforded increased duplex stabilities (*ca.* +0.5 °C per mod.) relative to the parent phosphoramidates **7A**.[34] The *arabino*-configured 2′-fluoro phosphoramidate **7C**, too, displayed increased duplex stabilities (*ca.* +0.5 °C per mod.) relative to phosphoramidates **7A**, and, despite the opposing *gauche* effect from the 2′-fluoro substituent, a preorganized C3′-*endo* conformation, as shown by the analysis of coupling constants for trimers, still accounts for the improved hybridization properties.[35]

The results obtained for the various phosphoramidates convincingly demonstrate that stereoelectronic preorganization into a C3′-*endo* furanose conformation can be applied to induce increased duplex stabilities. It should be mentioned, though, that a considerable sequence-variation has been observed and that, *e.g.* increases as small as +0.6 °C per mod. have been reported for fully modified unsubstituted phosphoramidates **7A**.[36] A point of concern with phosphoramidates is their more difficult incorporation into oligonucleotides with step-wise coupling yields in the range of 94–97%.[25,33,35]

Arabino nucleic acids and 2′-fluoroarabino nucleic acids (ANA and 2′-F-ANA)

The inversion of the configuration around C2′ of natural RNA gives arabino nucleic acids (**8A**, Fig. 8) which show decreased



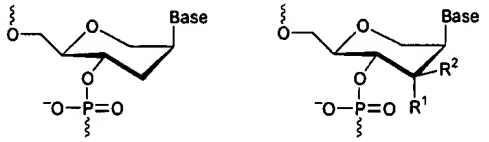
8A **8B**

Fig. 8 Structures of arabino nucleic acids.

duplex stabilities when hybridized to RNA while the corresponding 2′-fluoroarabino nucleic acids (**8B**), by contrast, enhance duplex stabilities by *ca.* +1.0 °C per mod.[37,38] The very interesting feature of both these modifications is that they were the first fully modified oligonucleotides with a chemically modified carbohydrate moiety to act as substrates for RNase H.[37–39] Their conformational similarity with natural DNA might account for this since duplexes, when hybridized to RNA, resemble an intermediate DNA–RNA duplex form as shown by CD spectroscopy.[38] The 2′-fluoro analogue **8B** adopts the O4′-*endo* furanose conformation when incorporated into DNA as shown by X-ray structure analysis.[40,41] In addition, molecular modelling[39,41] and NMR studies[39] of a fully modified 2′-F-ANA strand hybridized to RNA have confirmed an intermediate duplex form with a minor groove width intermediate between the minor groove widths of the standard A- and B-forms. The nuclease stability of the ANAs is improved compared with unmodified DNA though not as much as for the phosphorothioates.[37,39]

Hexitol nucleic acids (HNA)

Pyranose derivatives in general adopt one predominant conformation due to higher energy barriers for interconversion of ring conformers relative to furanose derivatives. To date, the 1′,5′-anhydrohexitol nucleic acids (HNA, **9A**, Fig. 9) and



9A **9B** R^1 = OH, R^2 = H; **9C** R^1 = H, R^2 = OH

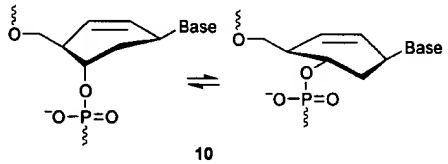
Fig. 9 Structures of hexitol nucleic acids.

derivatives constitute the most promising antisense candidates based on a pyranose moiety. Structurally, their substitution pattern makes them very good mimics of nucleotides pre-organized into an *N*-type (C3′-*endo*) furanose conformation thus yielding A-type duplexes when hybridized to RNA as shown by CD spectroscopy,[42] molecular dynamics simulations,[43] and NMR studies.[44] Fully modified HNA displays enhanced duplex stabilities (typically +3.0 °C per mod.)[45] while sequence-specific variations of increases of duplex stabilities ranging between +0.9[45] and +5.8[42] °C per mod. have been observed. HNA acts only as a very poor substrate for RNase H,[42] while its nuclease-resistance[45] still allows it to display antisense effects ascribed to a mechanism of steric blocking.[46]

Interesting derivatives of HNA are the 1,5-anhydro-2-deoxy-D-altritol nucleic acids with an additional axial hydroxy group in the 3′-position (**9B**, Fig. 9) which show slightly enhanced duplex stabilities relative to HNA.[47] The altritol nucleic acids were designed to increase the surface hydrophilicity of the duplex thereby favouring duplex formation by improved hydratization.[8] In contrast, the inversion of configuration at the 3′-position, affording the corresponding 1,5-anhydro-2-deoxy-D-mannitol nucleic acids, leads to significantly reduced stability of duplexes with RNA[48] and obviously imposes a restricted conformation unfavourable for duplex formation.

Cyclohexene nucleic acids (CeNA)

Noteworthy results have been obtained for the conformationally rather flexible cyclohexene nucleic acids (CeNA, **10**, Fig. 10)



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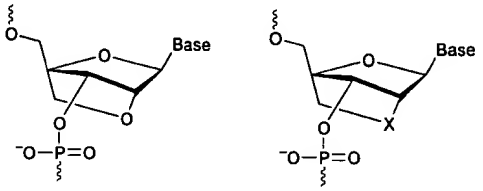
(*S*-type mimic) (*N*-type mimic)

Fig 10 Structure and conformational equilibrium of cyclohexene nucleic acids.

with a conformational equilibrium shifted towards *N*-type at the nucleoside level as shown by NMR and modelling studies (Fig. 10).[49] When incorporated into DNA strands, cyclohexene nucleotide monomers **10** induce increased duplex stabilities (+0.8 to +1.7 °C per mod.). Fully modified CeNA are nuclease-resistant and activate RNase H. The latter property establishes CeNA, next to ANA (Fig. 8), as the second fully modified oligonucleotide analogue with an altered carbohydrate part able to induce an RNase H cleavage of the target RNA strand.[50] This has been explained by the flexible conformational behaviour of the cyclohexane nucleotides allowing a $^2H_3$ (*S*-type mimic) conformation when hybridized to DNA and a $^3H_2$ (*N*-type mimic) conformation when hybridized to RNA as supported by CD spectroscopy, NMR studies of DNA duplexes with one cyclohexene nucleotide in each strand, and molecular dynamics simulations.[50]

Locked nucleic acids (LNA)

The synthesis of nucleosides and oligonucleotides containing furanose rings efficiently locked into a C3′-*endo* conformation has been accomplished by the introduction of an oxymethylene linkage between the C2′ and C4′ atoms generating the bicyclic locked nucleic acid (LNA, **11A**, Fig. 11). The incorporation of



11A **11B** X = NH; **11C** X = S

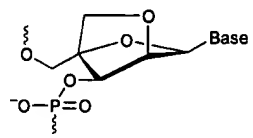
Fig. 11 Structures of locked nucleic acids (LNA), 2′-amino LNA and 2′-thio LNA.

one or more LNA monomers, or the use of fully modified LNA, induces unprecedented increases in duplex stabilities (typically +3.0 to +11.0 °C per mod.).[51–54] The preorganization into a C3′-*endo* furanose conformation has been demonstrated at the nucleoside level by X-ray crystallography[55] as well as NMR studies[51,52] and also by NMR studies at the oligonucleotide level both when hybridized to DNA[56–58] and RNA, the latter yielding the expected A-type duplexes.[59] LNA monomers in partly modified strands were shown to strongly preorganize flanking unmodified DNA nucleotides into *N*-type–C3′-*endo* furanose conformations and single-stranded LNA into an RNA-like conformation.[58,59] Fully modified[51] and mix-mer LNA consisting of alternating LNA and DNA monomers[60] have proved to be nuclease-resistant, and an antisense effect has been demonstrated in living rats.[60] LNA activates RNase H as gapmers[60] but not as fully modified strands.[60,61]

The LNA-type constitution leads in general to efficient C3′-*endo* preorganization and favoured duplex formation which has been demonstrated by the hybridization properties (+3.0 to +8.0 °C per mod.) obtained for the heteroatom derivatives 2′-amino-LNA[62] (**11B**, Fig. 11) and 2′-thio-LNA[63] (**11C**). A related C2′-methylene extended bicyclic nucleoside (thus containing a 2-oxapropylene linker between the O2′ and O4′ atoms), shown by X-ray crystallography and NMR studies at the nucleoside level likewise to be restricted into a C3′-*endo* furanose conformation,[64] induced less dramatic but still significantly increased duplex stabilities (+1.9 to +3.3 °C per mod.).[65]

α-L-LNA—a selected LNA stereoisomer

The inversion of the configuration around C3′ and/or C2′ and C4′ of LNA afforded three LNA stereoisomers which have been evaluated with respect to RNA binding.[66] Of these, 'α-L-LNA' (α-L-*ribo* configured LNA, **12**, Fig. 12) with inverted config-



12

Fig. 12 Structure of α-L-LNA.

uration around C2′, C3′ and C4′ compared to LNA, showed the most promising properties with respect to duplex formation as

shown by increased duplex stabilities of +4.3 to +5.7 °C per mod. for both partly and fully modified strands.[66–68] NMR and molecular modelling studies on duplexes of partly or fully modified α-L-LNA hybridized to DNA or RNA have established α-L-LNA as a DNA mimic and, in addition, that an α-L-LNA–RNA duplex adopts an overall conformation intermediate between the A and B form.[69–71] The possibility of RNase H activity has therefore been evaluated. Fully modified and mixmeric (consisting of alternating α-L-LNA and unmodified DNA nucleotides) α-L-LNA showed cleavage, albeit very slow, of the RNA targets at high enzyme concentrations.[61]

Summary and outlook

For efficient antisense action by the steric blocking mechanism, the use of antisense oligonucleotides containing preorganized furanose rings with *N*-type (C3′-*endo*, RNA-like) conformations and high binding affinities towards RNA target strands appears optimal. In contrast, the mechanism involving activation of RNase H demands analogues adopting *S*-type (C2′-*endo* or O4′-*endo*, DNA-like) furanose conformations.[2,41] It therefore appears impossible to take advantage of both mechanisms effectively with only one single nucleotide modification.

Despite the above points, the necessary armamentarium of chemically modified building blocks for development of efficient antisense therapeutics appears at hand. LNA, α-L-LNA and phosphoramidates, among others, offer excellent binding affinities towards RNA targets. In addition, as shorter strands (*e.g.* 10–14 nucleotides long) can be applied, good to excellent pairing selectivities are in general obtained. The binding affinity and pharmacokinetic properties are tuneable by combining these affinity-enhancing key nucleotide building blocks with, *e.g.* DNA, RNA, phosphorothioate-DNA, phosphorothioate-LNA, 2′-amino-LNA (or conjugated derivatives thereof), and 2′-*O*-alkyl-RNA nucleotides which have all been demonstrated to be applicable in DNA–LNA duplexes.[62,63,69] Whether the recruitment of RNase H is essential for efficient and general antisense action with these strongly RNA-binding nucleic acid mimics is currently not established. If RNase H activity turns out to be needed or beneficial, it is foreseen that the combination of one or more segment(s) of conformationally restricted or conformationally locked high-affinity *N*-type oligonucleotide(s) with a segment of nuclease-resistant, RNase H-activating *S*-type nucleotides with lower affinities should be ideal. A gap-mer structure is of course one possibility, but with the discovery of LNA and other extreme RNA-binders alleviating the need for binding cooperativity between the different segments, many other architectures appear possible. However, biological studies and pharmacological developments are needed to confirm these predictions.

The results obtained with ANA (**8**, Fig. 8), HNA (**9**, Fig. 9) and α-L-LNA (**12**, Fig. 12) are remarkable and need consideration. Firstly, very efficient RNA-binding is evidently possible for analogues based on a furanose skeleton with a non-natural stereochemical structure, or on an RNA-mimicking hexitol scaffold. Especially noteworthy is the dramatic RNA-binding of the α-L-*ribo* configured α-L-LNA in which the configuration at three out of the four chirality centers is inverted compared with RNA (or LNA). In addition, fully modified HNA and fully modified or mix-meric α-L-LNA have been demonstrated to induce very weak, but significant, RNase H activity. Results like these leave ample room for further curiosity-driven chemistry-based research on modified nucleic acids.

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